Nanoparticles Toxicity on *Escherichia coli*: Batch and Kinetic Approach

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NANOPARTICLES TOXICITY ON ESCHERICHIA COLI:
BATCH AND KINETIC APPROACH

BY

NELSON M. ANAYA-ARCHILA

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
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UNIVERSITY OF RHODE ISLAND
2016
DOCTOR OF PHILOSOPHY DISSERTATION

OF

NELSON M. ANAYA-ARCHILA

APPROVED:

Dissertation Committee:

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DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND
2016
ABSTRACT

Silver and dysprosium oxide are two examples of materials used for the manufacturing of nanoparticles with current and future commercial relevance, respectively. Silver nanoparticles (nAg or AgNPs) are one of the most commonly used nanomaterials in consumer products and medical applications due to their antimicrobial properties. Dysprosium oxide nanoparticles (nDy₂O₃) are gaining interest for biomedical applications because of their fluorescence and paramagnetic properties, which can be used as contrast agents in magnetic resonance analysis. However, the fate of nAg and nDy₂O₃ and their possible negative impacts on the environment and public health are growing concerns. Nanoparticles entering and accumulating in different environmental compartments will very likely interact with native bacteria in soil and aquatic environments.

There are knowledge gaps related to: the exposure of novel nanomaterials on microorganisms in different water chemistry conditions; the effect of reactor configuration to assess nanotoxicology; and the effect of the specific growth rate on the response of microorganisms exposed to nanoparticles.

In this study, nanoparticles toxicity on *Escherichia coli* (*E. coli*) was assessed under batch and continuous conditions, and evaluated their impacts on metabolic functions and cell structure such as, viability, membrane permeation, respiration, growth and changes in intracellular composition.
The results showed that several methodologies are needed to obtain a comprehensive understanding of the toxicological of the exposure of nanoparticles on microorganisms. At growing conditions, chemostat systems can provide a better assessment of the nanoparticle inhibitory effects on microorganisms in comparison to batch systems. However, there is not control of the contact time and the specific growth rate and contact time effects are combined. Longer term exposure and chronic studies are suggested to separate the growth rate effect from the contact time.

The data produced during this study is relevant to determine the real world implications on ecosystems and public health when the nanoparticles are released into the environment. With an understanding of the fate of nanoparticles in aqueous media, a more careful selection of toxicological methodologies and testing conditions can be made. This will allow for more accurate studies that measure the responses of microorganisms to the exposure of nanoparticles.
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PREFACE

This dissertation is in manuscript format. The first chapter is an introduction. The second chapter titled “Effects of dysprosium oxide nanoparticles on *Escherichia coli*” (2015) was published in Journal of Environmental Science Nano. Chapter 3 titled “Comparative study between chemostat and batch reactors to quantify membrane permeability changes on bacteria exposed to silver nanoparticles” (2016) has been published in Journal of Science of the Total Environment. Chapter 4 is a manuscript “Kinetic approach to quantify the response of *Escherichia coli* to nanoparticles exposure using chemostat reactors” and is prepared for submission. Supporting information for each manuscript is provided at the end of the respective chapter.
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CHAPTER 1

Introduction

Applications of nanoparticles have sharply increased during the last decade. More than 1,600 consumer nanoproducts have been introduced to the market since 2005 in fields such as electronic, automotive, biomedical, cosmetic, health, fitness and energy applications.

Silver and dysprosium oxide are two examples of materials used for the manufacturing of nanoparticles with current and future commercial relevance, respectively. Silver nanoparticles (nAg or AgNPs) are one of the most commonly used nanomaterials in consumer products and medical applications due to their antimicrobial properties. Dysprosium oxide nanoparticles (nDy$_2$O$_3$) are gaining interest for biomedical applications because of their fluorescence and paramagnetic properties, which can be used as contrast agents in magnetic resonance analysis.

However, the fate of nAg and nDy$_2$O$_3$ and their possible negative impacts on the environment and public health are growing concerns. Recent studies have shown that a large fraction nanoparticles used in consumer products are disposed in landfills or travel through wastewater treatment plants to end up in soil and water because of the inability of wastewater and sludge treatment facilities to retain and remove these nanoparticles. Nanoparticles entering and accumulating in different environmental compartments will very likely interact with native bacteria in soil and aquatic environments.
The inhibitory effect of nanoparticles on bacteria depends on physicochemical properties of the nanoparticles (e.g., charge, aggregation, cell-nanoparticle ratio and dissolution) which can differ among each other due to changes in size, charge, coating agent, manufacturer, reagents used during the synthesis and at the same time the water chemistry conditions influence the nanoparticle performance and bacteria-nanoparticle interaction. Release of ions from nanoparticles to bacteria promotes lysis, which makes nanoparticles, including silver a widely used antimicrobial agent. AgNPs may serve as a medium to deliver Ag$^+$ more effectively (binding and reduced bioavailability by common natural ligands to the bacteria membrane and cytoplasm is less susceptible), whose proton motive force would reduce the local pH (close to pH 3.0) and improve Ag$^+$ release$^9$. The effect of nanoparticles on bacteria depends of variables including the exposure time and concentration. Chronic toxicity can be assessed using long term low exposure concentrations during several generations of bacteria$^{10}$. Otherwise, acute tests correspond to a single dose exposure in a short period of time, which can mimic accidental manufacturing spill scenarios$^{10}$. Likewise, metabolic activity also can affect bacteria response to stress conditions. Populations of bacteria growing rapidly had a harder time to adapt than those growing slowly, but this not have been proven for nanoparticles. Furthermore, a careful selection of appropriate toxicological methodologies is required to obtain nanotoxicological data with high accuracy.

In this study, the nanoparticles toxicity on *Escherichia coli* (*E. coli*) was assessed under batch and continuous conditions, and evaluated their impacts on metabolic
functions and cell structure such as, viability, membrane permeation, respiration, growth and changes in intracellular composition.

The objectives of this dissertation work are:

1) Evaluate the effect of nDy₂O₃ on the metabolic activity of *E. coli* at relevant environmental conditions such as: carbon sources and water chemistry conditions. These results could elucidate toxicological mechanisms not described in the current scientific literature and identify correlations between the nutritional stage of the bacteria and nDy₂O₃ concentrations on the toxicity effect.

2) Compare batch and chemostat systems to assess the toxicity of nAg on *E. coli* based on membrane permeability. Bioreactor experiments, coupled with Langmuir film balance analysis of lipid monolayers formed using *E. coli* membrane extracts can be used to assess nanoparticles exposure. The rate of substrate utilization and product formation are dependent on the growth conditions and can influence the nature and magnitude of the effect of nanoparticles on the cell membrane, and thus, bacterial metabolism.

3) Quantify the inhibitory effect of nAg on components of bacteria at different contact times and growth rates. The variations of specific functional groups in biomolecules allow to identify changes in the total composition of bacteria exposed to nAg through chemostats coupled with Fourier transform infrared (FTIR) and study the effect of the physiological stage of the bacterial population.

These studies produced relevant data about the implications on the environment and public health when the nanoparticles are released into the environment. In addition, a better understanding for the selection of toxicological methodologies with
the purpose to increase the accuracy of future nanotoxicological studies will be assessed.
References


CHAPTER 2

Effects of dysprosium oxide nanoparticles on Escherichia coli

By

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Abstract

There is increasing interest in the study of dysprosium oxide nanoparticles (nDy$_2$O$_3$) for biomedical applications due to their fluorescent and paramagnetic properties. However, the fate of nDy$_2$O$_3$, and their effects on natural biological systems, are a growing concern.

This study assessed the toxicity of nDy$_2$O$_3$ on *Escherichia coli* for concentrations between 0.02 and 2 mg/L, exposed to three concentrations of NaCl (8,500; 850 and 85 mg/L) and three glucose concentrations (35, 70, 140 mg/L). The ranges of these variables were selected to cover manufacturer recommendations of analytical methodologies for toxicity assessment, environmental and industrial nDy$_2$O$_3$ effluent concentrations, and metabolic activity. Two array-based toxicity techniques were used to evaluate the 27 combinations of conditions. Fluorescent dyes (Live/Dead) and respirometric assays were used to measure the undisturbed cell membrane (UCM) and remaining respiration percentage (RRP), respectively.

Respirometric tests showed a higher toxic effect than Live/Dead test assays, indicating that metabolic processes are more affected than the physical structure of the cell by exposure to nDy$_2$O$_3$. After exposing the bacteria to concentrations of 2.0 mg/L uncoated nDy$_2$O$_3$ for 2 hrs at 85 mg/L of NaCl and 140 mg/L of glucose, the RRP and UCM decreased to 43% and 88%, respectively. Dysprosium ions (Dy$^{3+}$) toxicity measurement suggested that Dy$^{3+}$ was the main contributor to the overall toxicity.

2.1 Introduction
Gadolinium, holmium and dysprosium belong to the lanthanide oxide-based nanoparticles (LnONps), which have acquired more relevance in recent years in regard to the locating, diagnosing and treating of diseases\textsuperscript{1-3}. LnONps have unique paramagnetic properties that allow greater spatial and temporal resolution through a higher signal-to-noise ratio. These properties play a fundamental role in acquiring and enhancing the contrast in T\textsubscript{1} or T\textsubscript{2} magnetic resonance images (MRI)\textsuperscript{4,5}. Due to the higher sensitivity provided by the LnONps, the MRI contrast is improved and the T\textsubscript{1} or T\textsubscript{2} relaxation times are discriminatorily shortened in the region of interest\textsuperscript{6}.

Dysprosium oxide nanoparticles (nDy\textsubscript{2}O\textsubscript{3}) have recently received increasing attention due to their potential applications in the biomedical field\textsuperscript{4,5} including cancer research, new drug screening, and the delivery of drug applications\textsuperscript{2,7,8}. However, the fate of nDy\textsubscript{2}O\textsubscript{3} and their effects on natural biological systems are growing concerns\textsuperscript{9}. nDy\textsubscript{2}O\textsubscript{3} will enter into aquatic and land environments through wastewater treatment facility effluent and wastewater sludge due to an inability to retain and or remove these nanoparticles completely\textsuperscript{10}. Moreover, the release of nDy\textsubscript{2}O\textsubscript{3} into land environments from agricultural applications could transport nanoparticles to surface waters via stormwater runoff and to groundwater via infiltration through the soil\textsuperscript{10,11}.

Previous studies have provided limited insight into the toxic effects of nDy\textsubscript{2}O\textsubscript{3} and Dy ions on natural systems. Kattel et al.\textsuperscript{12} investigated the in vitro toxicity effect of ultra-small spherical dysprosium oxide and dysprosium hydroxide nanorods\textsuperscript{5}. Both nanoparticles were coated with D-glucuronic acid and exposed to DU 145 and NTC 1469 cell lines. These studies showed that the nanoparticles were not toxic to the human cells for concentration values ranging from 0 to 37.3 mg/L.
Harper et al.\textsuperscript{13} tested 11 types of metal oxide nanoparticles, including nDy\textsubscript{2}O\textsubscript{3}, and found that high mortality of embryonic zebrafish was observed when they were exposed to 250 mg/L of nDy\textsubscript{2}O\textsubscript{3} for 5 days of continuous waterborne conditions. In addition, concentrations of 250 mg/L for nDy\textsubscript{2}O\textsubscript{3} produced morphological malformations of the zebrafish’s jaw and eyes.

Toxicological assessment of nanoparticles can be studied in terms of their impact on metabolic functions and cell structure such as cell viability, membrane permeation, growth and respiration. Live/Dead assay (BacLight viability kit) is a commonly-used method to measure cell viability\textsuperscript{14} and membrane permeation on bacteria through the integrity of cell membranes. The manufacturer of the reagents used for the Live/Dead test recommends that experiments and samples have to be prepared in specific water chemistry conditions (8,500 mg/L of NaCl) to avoid a decrease in staining efficiency\textsuperscript{14}. Previously, the metabolic activity of bacteria has been measured using a traditional respirometric bottle test (RT). Water chemistry conditions with monovalent and divalent cations have been successfully used in the range of 10 to 1,000 mg/L; however, high concentrations of glucose (in the order of 300 mg/L) and bacteria (in the order of 10\textsuperscript{9} CFU/mL) were required to quantify a toxic response\textsuperscript{15}. This type of test can be used to measure the interaction and effect of nanoparticles on microorganisms. Nevertheless, each methodology required its own range of optimal conditions, which makes it a complex process to assess toxic effects when identical water chemistry conditions are used. This is highly relevant to the evaluation of the toxic effect of nanoparticles, since the physicochemical properties of the nanoparticles (e.g., charge, aggregation, cell-nanoparticle ratio and dissolution) can differ among
each other and also their properties are influenced by the physicochemical characteristics of the aqueous solution.

In this study, we propose to evaluate the use of array-based dyes methods in identical water chemistry conditions and observe the effect of nDy$_2$O$_3$ and exposure on $E. \text{coli}$ metabolic activity and structural integrity of $E. \text{coli}$ under variable water chemistry conditions.

2.2 Materials and Methods

2.2.1 Materials

A non-pathogenic wild strain of $E. \text{coli}$ (IDEXX laboratory) was selected for this study. $E. \text{coli}$ is a Gram-negative bacterium that has been found to be metabolically active in saline solution without growth$^{16}$ and has been extensively studied in nanotoxicological research.$^{15,17,18}$ Reagents used to prepare the growth media for the bacteria — sodium chloride (NaCl), yeast extract, and tryptone — were purchased from Sigma Aldrich. Glucose was purchased from Sigma Aldrich and used as received. Tetrazolium dye (Redox Dye Mix A) was purchased from Biolog and used to measure the respiratory responses of $E. \text{coli}$. Cell membrane permeation was measured using SYTO 9 and propidium iodide; both reagents were purchased from Invitrogen.

2.2.2 Methods

Hydrodynamic diameter and zeta potential were measured by Malvern Zetasizer Nano ZS, ZEN 3600, dynamic light scattering (DLS). Data was collected at 0.25 hrs and at 2 hrs after nanoparticles exposure to bacteria to differentiate the effect of
aggregation of nanoparticles. Shape characterization of the nanoparticle was obtained by using JEOL JEM-2100 LaB6 transmission electron microscope (TEM) imaging. Ionic release from nDy$_2$O$_3$ for each condition was quantified as per Liu and Hurt$^{19}$ using centrifugal ultrafilter devices (ultra-4,3K) purchased from Amicon. Inductively-coupled plasma spectroscopy (ICP-OES optima 3100, Perkin Elmer) was used to measure the concentration of nDy$_2$O$_3$ and Dy ions before and after the contact times established for each of the water chemistry conditions tested. Samples were digested in nitric acid (2% v/v, HNO$_3$) before analysis.

Growth media consisted of 10 g/L NaCl, 5 g/L yeast extract, and 10 g/L tryptone. After the solution was prepared, it was autoclaved and then inoculated with *E. coli*. *E. coli* was grown for 12 hrs in a culture media at 37°C. Bacteria were harvested during the logarithmic growth phase and centrifuged at 2000 rpm (751 g) for 0.25 hrs. The supernatant was discarded and the pellet re-suspended in the respective NaCl solution$^{15}$. *E. coli* concentration was fixed to OD$_{670}$ (optical density at a wavelength of 670nm) of 2.68 using the respective NaCl solution. *E. coli* solutions were refrigerated at least 30 min prior to inoculation into the microplate in order to decrease the metabolism of the bacteria. This allowed for a homogenous metabolic reaction to the glucose.

Three NaCl concentrations were selected: 85 mg/L, 850 mg/L and 8500 mg/L (ionic strength 1.45 mM, 14.5 mM and 145 mM, respectively). The lowest concentration represents the ionic strength commonly found in surface waters,$^{20}$ while the highest NaCl concentration was selected based on the manufacturer’s recommendations of the Live/Dead test$^{14}$. The middle value was chosen for a three-tier
comparison. Glucose concentrations in the range of 35 mg/L to 140 mg/L were used as a carbon source to evaluate nDy$_2$O$_3$ toxicity under different aerobic metabolic levels. Preliminary experiments were carried out to determine the glucose concentrations that inhibit the *E. coli* metabolic functions. The glucose concentration of 35 mg/L was the lowest limit of respiratory detection, and 140 mg/L was below the concentration (210 mg/L) that produces an inhibitory effect on *E. coli*.

Three concentrations of non-coated Dy$_2$O$_3$ nanoparticles — 0.02 mg/L, 0.2 mg/L and 2.0 mg/L — were used to simulate not only environmental concentrations (0.02 mg/L), but also accidental spill scenarios (2.0 mg/L). Non-coated Dy$_2$O$_3$ nanoparticles were prepared based on the method used by Kattel et al.$^{12}$.

Table 1 shows the 27 conditions and the blanks tested in this study for the respirometric and Live/Dead tests using the synergy TM MX microplate reader. Blanks without nDy$_2$O$_3$, glucose and NaCl were analyzed for each scenario.

Table 1 Condition Matrix tested using respirometric and live/dead tests. Samples were run in quadruplicate. Glucose, nDy$_2$O$_3$ and bacteria were prepared at the same NaCl concentration. * represents blanks for each condition.

<table>
<thead>
<tr>
<th>NaCl [mg/L]</th>
<th>Glucose [mg/L]</th>
<th>nDy$_2$O$_3$ [mg/L]</th>
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<td>0*</td>
<td>0*</td>
<td>0.02, 0.2, 2</td>
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### 2.2.3 Toxicity tests

**2.2.3.1 Respirometric test.** Cell respiration in non-growing conditions was quantified through the reduction of tetrazolium dye\(^{21,22}\). When a carbon source is consumed, metabolic activity transports electrons from the carbon source to the electron transport chain in the cell membrane where the tetrazolium dye is reduced, which in turn produces a purple colour\(^{23,24}\). The purple colour can be quantified in terms of absorbance using a microplate reader at a wavelength of 590 nm\(^{25}\). The plate was prepared first by adding NaCl, then glucose, nDy2O3, tetrazolium dye, and finally a bacteria solution were directly inoculated into a Blank 96 half-area well microplate to achieve the required concentrations. The final volume within each well was 100 µL, mixed thoroughly by pipetting at least 10 times. Glucose, nDy2O3, and cell solutions were all prepared in the respective saline solution. Experiments were run for 2.5 hrs.
and readings were recorded every 0.25 hrs after every horizontal shaking (medium setting) performed by the microplate reader. Data were analyzed at 0.25 and 2 hrs. The two-hour interval was selected in order to distinguish the effect between nanoparticles before and after aggregation. Aggregation increased drastically after 2 hrs, particularly for those conditions with high concentrations of NaCl. Higher exposure time could increase toxicity effect; however, for time periods longer than 2hrs, the toxicity results will not be because of the dysprosium nanoparticles but because of the dysprosium microparticules. The microplates were incubated at 25°C. A detailed description of the microplate set-up is presented in the Supplemental Information section.

The microplate included nDy$_2$O$_3$ blank, and background correction sections to evaluate the interaction between nanoparticles and tetrazolium dye. Lack of coloration in the nDy$_2$O$_3$ blank section indicated that no interaction was observed between nDy$_2$O$_3$ and the tetrazolium dye. For the background (absorbance of nDy$_2$O$_3$) correction, the value obtained in the respective well, was subtracted from the experiment values and also served as a secondary control to confirm that experimental conditions had no reducing effect on the tetrazolium dye without the presence of bacteria.

Each plate has been set up in quadruplicate wells for each condition, and the plates were run in duplicate to quantify the percent of remaining respiration (PRR). The PRR (Eq. 1) is the ratio of slopes between bacteria exposed to nDy$_2$O$_3$ and the blank bacteria (samples containing bacteria that were not exposed to nDy$_2$O$_3$) at a specific nDy$_2$O$_3$ concentration from the absorbance-time graph.
\[
\text{PRR} = \frac{P_t}{P_c} \times 100\% \quad \text{Eq. 1}
\]

Where,

- \( P_t \) = slope from absorbance-time graph for bacteria exposed to nDy\(_2\)O\(_3\)
- \( P_c \) = slope from absorbance-time graph for bacteria control without nDy\(_2\)O\(_3\)

2.2.3.2 Live/dead test. The cell membrane permeation of \( E. \ coli \) was determined using the BacLight kit (propidium iodide and SYTO 9) with the microplate reader. Propidium iodide becomes intercalated to the DNA within cells, and indicates bacteria that have a damaged membrane. SYTO 9, on the other hand, indicates intact cell membranes\(^{11} \). A stain solution composed of SYTO 9 and propidium iodide fluorescent nucleic acid stains was mixed at a 1:1 (v/v) ratio with a subsequent dilution in DI water (12μL of stain mixed solution in 2 mL of DI water). Suspension mixtures of NaCl, glucose, nDy\(_2\)O\(_3\), and bacteria were added into separate wells of a 96 well flat-bottom black microplate to achieve the required concentrations. The plate was incubated at 25°C for 2.0 hrs, during which horizontal shaking (medium setting) was performed by the microplate every 0.25 hrs. After incubation, 100 μL of mixed stain solution was added and mixed thoroughly by pipetting at least 10 times for each well. Before reading with the microplate, 0.25 hrs of additional incubation was required in the dark at room temperature. A detailed description of the microplate setup is presented in the Supplemental Information section. Each plate contained quadruplicate wells for each condition, and each plate was run in duplicate to quantify the undisturbed cell membrane (UCM). The UCM (Eq. 2) is the green/red fluorescence ratio between bacteria exposed to nDy\(_2\)O\(_3\) and the blank bacteria (bacteria not exposed
to nDy$_2$O$_3$) at given nDy$_2$O$_3$ concentration. Data was analyzed at 0.25 and 2 hrs to differentiate the effect of aggregation of nanoparticles.

\[ \text{UCM}=\frac{P_t}{P_c} \cdot 100 \]  

Eq. 2

Where,

- $P_t =$ Green/red fluorescence ratio for bacteria exposed to nDy$_2$O$_3$
- $P_c =$ Green/red fluorescence ratio for bacteria control without nDy$_2$O$_3$

2.2.3.3. Toxicity tests for nDy$_2$O$_3$ ion release. Additional experiments using the respective ions concentration, released at the highest concentration of nDy$_2$O$_3$, were performed. These experiments allow us to determine the contribution of Dy ions to the overall toxicity on *E. coli*. Similar method was used to prepare the plate, but dysprosium ions were used instead of nDy$_2$O$_3$.

2.2.4 Statistical analysis

The results from each data set were analyzed with SAS statistical software, version 9.1.2. A generalized linear mixer model (GLIMMIX) was used to identify statistical differences among glucose, NaCl, and nDy$_2$O$_3$ concentrations because the response was not necessarily normally distributed. A p value of less than 0.05 was considered to indicate significant difference.

2.3 Results and Discussion

2.3.1 Physiochemical characterization of nDy$_2$O$_3$

2.3.1.1 Size and zeta potential without bacteria. Uncoated nDy$_2$O$_3$ characterization
consisted of size and zeta potential measurements in two of the three water chemistry conditions. Tests with 8500 mg/L NaCl were discontinued due to interference between tetrazolium dye and NaCl. Details will be explained in Section 3.2. Figure 1 shows size (a) and zeta potential (b) measurement at 0, 0.25 and 2.0 hrs for the highest concentration of 2.0 mg/L of nDy₂O₃. nDy₂O₃ sizes from 0 to 0.25 hrs at all water chemistry conditions were in the range of 75 to 150 nm; however, size measurements at 2 hrs showed an increase in nDy₂O₃ particle size, indicating aggregation. It was observed that aggregation increased when the ionic strength was higher (850 mg/L NaCl), and also at all glucose concentrations. This may be attributed to the absence of a coating agent on the nanoparticles surface and lower repelling forces as a result of high ionic strength in the solution. Zeta potential measurements (Figure 1b) showed the instability of nDy₂O₃ with surface charge measurements ranging between 6 and 23 mV in 85 and 850 mg/L of NaCl during 2 hrs of contact time.

2.3.1.2. Shape and pH. TEM imaging confirmed the shape of the nDy₂O₃ to be spherical (Figure 2). Nanoparticles were found to have an average size of 74.8 ± 5 nm. Changes in H⁺ ions before and after nanoparticle exposure were recorded periodically with a pH meter. The pH measurements at t = 0 hrs and t = 2 hrs ranged from 5.5 to 6.2, indicating that pH did not function as an additional stress on bacteria performance. Moreover, there was no statistically significant change.

2.3.1.3. Ion release. Ion release experiments were conducted for the highest concentration of nDy₂O₃, (2.0 mg/L), which was the most toxic condition for E. coli.
Dy ions were measured in all water chemistry and glucose conditions to determine the amount of dissolution over time. Table 2 shows a sudden increase in Dy ions measured at 0 hrs and 0.25 hrs, with a plateau occurring at 2.0 hrs.

Figure 1 Size (a) and zeta potential (b) measurements in samples containing 35 mg/L, 70 mg/L, and 140 mg/L glucose, and 2.0 mg/L nDy₂O₃. Measurements were taken at 0 hrs, 0.25 hrs and 2 hrs.
Figure 2 nDy$_2$O$_3$ TEM image shows the spherical nanoparticles with average diameter of 74.8 nm.

Table 2 Ion release concentration for 2.0 mg/L nDy$_2$O$_3$ in samples containing 85 mg/L and 850 mg/L NaCl, and 35 mg/L, 70 mg/L and 140 mg/L glucose. Measurements were taken at 0 hrs, 0.25 hrs and 2.0 hrs.

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2.3.2 Toxicity tests

Respirometric tests at 8,500 mg/L of NaCl detected interference between tetrazolium dye and NaCl. Bacteria at 85 and 850 mg/L of NaCl without stress conditions maintained an absorbance value after the dye was reduced. On the other hand, bacteria at 8,500 mg/L showed the opposite behaviour (see SI). Interference with glucose was discarded because Live/Dead tests showed no problems under similar condition. Although, absorbance measurements could not be obtained at high NaCl concentration, it is known that *E. coli* may survive under high ionic strength conditions similar to those presented in this study$^{26-28}$. 

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2.3.2.1 Respirometric test. The PRR compares the slope values representing respiration of the remaining bacteria after nDy₂O₃ exposure with the slope values of the control wells. In the control wells, bacteria were not exposed to nDy₂O₃.

Figure 3 shows the PRR for all water chemistry conditions tested at 0.25 and 2 hrs. For 0.25 hrs, no toxicity effect was observed for most glucose concentrations of 35 and 70mg/L, except for 35 mg/L of glucose and 0.02mg/L of nDy₂O₃. This could be due to the fact that the nDy₂O₃ contact time was not enough to produce a significant toxicity effect in the bacteria. After 2.0 hrs, there was an increase in toxicity for some water chemistry conditions. For 2.0 mg/L nDy₂O₃, the toxicity effect was more pronounced at all glucose and NaCl concentrations. The greatest toxicity effect (PRR=43%) was observed at 85 mg/L NaCl and 140 mg/L glucose. The high PRR values may be associated with the metabolic activity at high carbon concentration levels and more stable nanoparticles at low concentrations of NaCl. *E. coli* increases the carbon mineralization at glucose concentration of 140 mg/L, where Dy ions can be transported inside the bacteria that are coupled to an essential metal uptake by the cells²⁹.

2.3.2.2 Live/dead test. The undisturbed cell membrane (UCM) results are shown in Figure 4. Live/Dead experiments detected a very low toxicity effect of nDy₂O₃ on *E. coli* under the same conditions used for the respirometric microarray tests. The results showed no significant membrane permeation, which means minimal (UCM= 94.9) to no physical damage to *E. coli* occurred at nDy₂O₃ concentrations ranging from 0.02 to 0.2 mg/L. Only at high concentrations of nDy₂O₃ (2 mg/L) and at ionic strength (850
mg/L NaCl), a slight toxicity effect (UCM= 88.5) was observed on bacteria. However, it was less pronounced compared to the toxicity effect results obtained for the respirometric test. The increasing concentrations of glucose seem to show no constant trend. Thus, glucose concentration could not have influence the toxicity effect results.

2.3.2.3 Effect of Dy ions on E. coli Toxicity. Additional experiments were performed to determine the contribution of Dy ions to the overall toxicity on E. coli. The tests were performed using the respective ions concentration released at the highest concentration of nDy₂O₃ (table 2), as that exhibited the highest toxicity effect on bacteria. The results showed that cell viability was predominantly lost due to interactions of Dy⁺³ ions with E. coli rather than nDy₂O₃ (Figure 5). This suggests that nDy₂O₃ could have caused damage to the cell membrane, and Dy⁺³ could have entered into the cell and disturbed intracellular activities, as previously presented.

2.3.2.4 Comparison between toxicology methodologies

This study performed a comparison between two toxicological tests using nDy₂O₃ on E. coli. It was found that for both methodologies, and at the same conditions, nDy₂O₃ is most toxic at high concentrations [2.0 mg/L]. The respirometric test showed a more prominent response (PRR=43%) compared to Live/Dead (UCM=88%) test. This indicates that metabolic responses are more sensitive to toxicity than cell physiology when bacteria are exposed to nDy₂O₃.

Statistical analysis (for details, see Supplemental Information section) confirmed a correlation — first with the nDy₂O₃ concentrations (p<0.0001), then with NaCl concentrations (p=0.0040) and finally with glucose concentrations (p=0.0074) on the
exposure response to nDy2O3 for the metabolic activity of the cell. For both methods, nDy2O3 concentration was the most influential variable and determined the magnitude of the exposure response. In addition, a strong correlation was observed when the combined effect between NaCl and nDy2O3 (p=0.0148) was analyzed. This is consistent with the results obtained, which showed higher toxicity effect in conditions with low NaCl and high nDy2O3 concentrations. Also, NaCl and glucose were analysed simultaneously showed a significant correlation with a p value of 0.0566. Similar trend was observed in the high toxicity effect of nDy2O3 on E. coli when bacteria were more active at high glucose concentrations and nDy2O3 more stable at low concentrations of NaCl.
Figure 3 Percent remaining respiration (PPR) of *E. coli* under different water chemistry conditions, (NaCl 85 mg/L and 850 mg/L), and glucose concentration (35 mg/L, 70 mg/L and 140 mg/L). PPR values were calculated for three concentrations of nDy$_2$O$_3$, 0.02 mg/L, 0.2 mg/L and 2.0 mg/L at (a) 0.25 hrs and (b) 2.0 hrs. Values are mean from 4 wells and duplicate plates.
Figure 4 Undisturbed cell membrane (UCM) of *E. coli* under different water chemistry conditions, (NaCl 85 mg/L and 850 mg/L), and glucose concentration (35 mg/L, 70 mg/L and 140 mg/L). UCM values were calculated for three concentrations of nDy$_2$O$_3$, 0.02 mg/L, 0.2 mg/L and 2.0 mg/L at (a) 0.25 hrs and (b) 2.0 hrs. Values are mean from 4 wells and duplicate plates.
Figure 5 Toxicity effect of dysprosium ions ($\text{Dy}^{3+}$) and $\text{nDy}_2\text{O}_3$ under different water chemistry conditions: (a) 850 mg/L and b) 85 mg/L) and glucose concentration (35 mg/L, 70 mg/L, and 140 mg/L) at the highest concentration of ions released after 2 hrs for 2 mg/L of $\text{nDy}_2\text{O}_3$.

2.4 Conclusion

The results showed that respirometric and permeation membrane tests can be used to provide a comprehensive assessment of nanoparticle toxicity on microorganisms. This study evaluated the performance of two toxicity methodologies: the Live/Dead assay to evaluate the membrane permeation, and the respirometric assay to evaluate the metabolic activity of bacteria. The respirometric microarray test proved to be more sensitive than the Live/Dead test in measuring nanoparticle toxicity.

With an understanding of the fate of nanoparticles in aqueous media, a careful selection of appropriate toxicological methodologies can be made to improve the accuracy of future nanotoxicological studies.

Acknowledgements
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References


Effects of Dysprosium Oxide Nanoparticles on *Escherichia coli*

N. M. Anaya, F. Solomon and V. Oyanedel-Craver

Supplemental information

Conditions and toxicity tests

Figure S1: Conditions and toxicity tests analyzed in this study

**Respirometric Plate Map**

The various sections, labelled within the microplate, represent the quality control/quality assurance (QC/QA) measures implemented in each microplate experiment constructed. Sections of the microplate, e.g., nDy$_2$O$_3$ blank, bacteria blank and background correction, represent sections to detect false positives, since no coloration should develop in these wells during the testing period. No coloration in the nDy$_2$O$_3$ blank section indicates no reduction interactions between nDy$_2$O$_3$ and the tetrazolium dye that would produce colour. The Bacteria Blank section should remain colourless; because there is no glucose present therefore electron transfer is not
possible. To account for background absorbance of nDy$_2$O$_3$, the Background Correction section was subtracted from the Experiment section; and it also served as a secondary control to further confirm that experimental conditions had no reducing effect on the tetrazolium dye without the presence of bacteria. Lastly, the Reference condition contained no nDy$_2$O$_3$ and was the experimental control against which all experimental values were compared.

Figure S2: Respirometric plate map used to conduct the nDy$_2$O$_3$ toxicity experiments. Each plate tested one water chemistry condition and one concentration of glucose while concentration of nDy$_2$O$_3$ were varied

Respirometric results for 8,500mg/L NaCl and 70mg/L of glucose

Figure S3: nDy$_2$O$_3$ toxicity on E. coli at 8,500 mg/l of NaCl

Statistical Analysis

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Figure S4: Variability analysis for Respirometric tests for the toxicity of nDy$_2$O$_3$ in two water chemistry conditions (85mg/L and 850 mg/L) using SAS
CHAPTER 3

Comparative study between chemostat and batch reactors to quantify membrane permeability changes on bacteria exposed to silver nanoparticles

By

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Abstract

Continuous and batch reactors were used to assess the effect of the exposure of casein-coated silver nanoparticles (AgNPs) on *Escherichia coli* (*E. coli*). Additionally, *E. coli* membrane extracts, membrane permeability and Langmuir film balance assays were used to determine integrity and changes in lipid composition in response to AgNPs exposure.

Results showed that batch conditions were not appropriate for the tests due to the production of exopolymeric substances (EPS) during the growth phase. After 5 hours of contact between AgNPs and the used growth media containing EPS, the nanoparticles increased in size from 86 nm to 282 nm reducing the stability and thus limiting cell-nanoparticle interactions. AgNPs reduced *E. coli* growth by 20% at 1 mg/L, in terms of Optical Density 670 (OD$_{670}$), while no effect was detected at 15 mg/L. At 50 mg/L of AgNPs was not possible to perform the test due to aggregation and sedimentation of the nanoparticles. Membrane extract assays showed that at 1 mg/L AgNPs had a greater change in area (−4.4 cm$^2$) on bacteria compared to 15 mg/L (−4.0 cm$^2$). This area increment suggested that membrane disruption caused by AgNPs had a *stabilizing/rigidifying effect* where the cells responded by shifting their lipid composition to more unsaturated lipids to counteract membrane rigidification.

In chemostats, the constant inflow of fresh media and aeration resulted in less AgNPs aggregation, thus increased the AgNPs-bacteria interactions, in comparison to batch conditions. AgNPs at 1mg/L, 15mg/L, and 50mg/L inhibited the growth (OD$_{670}$ reduction) by 0%, 11% and 16.3%, respectively. Membrane extracts exposed to 1mg/L, 15mg/L, and 50mg/L of AgNPs required greater changes in area by -0.5cm$^2$,
2.7cm² and 3.6cm², respectively, indicating that the bacterial membranes were disrupted and bacteria responded by synthesizing lipids that stabilize or strengthen membranes.

This study showed that the chemostat is more appropriate for the testing of nanotoxicological effects when testing bacteria at growing conditions.

3.1 Introduction

Silver nanoparticles (AgNPs) are one of the most commonly used nanomaterials in consumer products due to their antimicrobial properties (Kasaraneni et al., 2014; Schifman et al., 2015). While the antimicrobial properties of AgNPs are beneficial for several medical applications (Prabhu and Poulose, 2012; Li et al., 2008), the accidental release of them can negatively affect bacterial populations responsible to important biogeochemical cycles (Marambio-Jones and Hoek, 2010; Panyala et al., 2008). Several studies have demonstrated the antimicrobial properties of AgNPs and the critical role of membrane integrity; however, changes in the composition and properties of bacterial membranes due to AgNPs exposure, is not yet completely understood (Guzmán et al., 2012).

Cell membrane acts as a permeability barrier to the cytoplasm and is able to regulate the transport of macro- and micro- nutrients from the media to the cytoplasm as well as the osmotic pressure through the plasma membrane. The osmotic pressure influences the integrity and hydration of cells and their intracellular compartments. Inflowing of water and swelling is governed by a decrease in external osmotic
pressure (Wood, 2015), whereas an increase in osmotic pressure results in outflowing of water and dehydration. However, water fluxes coming simultaneously from opposite directions can disturb several cellular properties, including cell volume, turgor pressure, strain and cytoplasmatic membrane tension. Attenuation of water fluxes, by the accumulation or release of solutes, is one mechanism by which cells will respond to changes in external osmotic pressure (Wood, 2015).

AgNPs can damage bacterial membrane via three mechanisms. First, the electrostatic interaction between cell membranes and nanoparticles can interrupt transmembrane electron transfer, and produce break formation (pit formation). Through this mechanism, AgNPs can also penetrate into the cell membrane producing an increase in the permeability, resulting in an uncontrolled plasma-membrane transport and even leading to cell death (Prabhu and Poulose, 2012). Secondly, AgNPs can release silver ions (Ag⁺) through cooperative oxidation with both protons and dissolved O₂ (Liu and Hurt, 2010). Ag⁺ can be transported in the bacterial membrane by the potential disruption of nanoparticles to the cell wall and membrane. Bacterial membrane permeability can be affected by the Ag⁺ mechanism described above and cause the release of lipopolysaccharides (LPS) and membrane proteins (Losasso et al., 2014). Moreover, Ag⁺ can cause the release of phosphate, mannitol, succinate, proline and glutamine from the cytoplasm and disrupt the respiration cycle by inhibiting the uptake of phosphorous, thus impairing the formation of energy-regulating compounds such as nicotinamide adenine dinucleotide (NADH) or damaging molecules, such as DNA (Rai et al., 2012).
Finally, the interaction between Ag\textsuperscript{+} and thiol groups in proteins, in addition to inactivating the respiratory enzymes, can lead to the production of undesirable compounds, such as reactive oxygen species (ROS) (Li et al., 2008). Intracellular oxidative stress can then occur as a result of high amounts of ROS, which can cause changes in the permeability of the cell membrane, protein structure, mitochondrial activity, and DNA replication (Manke et al., 2013; Eckhardt et al., 2013; Prabhu and Poulse, 2012).

Bioreactors are used to grow bacteria in continuous or batch mode. Previous studies have provided insight into the potential use of continuous reactors (chemostats) to assess stress conditions on bacteria. The effect of pH, osmotic stress, antibiotic resistance, and temperatures on bacteria have been studied extensively using chemostats (King et al., 2006) (Leenheer and Cogan, 2008). In chemostats, bacteria response in terms of cell growth and adaptation can be studied under single and multiple conditions, such as competition with nutrient recycling and antibiotic treatment (Lin et al., 2012; Ziv et al., 2013; Miller et al., 2013; Gresham and Hong, 2015). Comparatively, batch reactors have been used broadly to quantify the antimicrobial properties of nanoparticles in terms of their impacts on metabolic functions and cell structure such as, viability, membrane permeation, growth and respiration (Anaya et al., 2015; Mirzajani et al., 2011; Roe et al., 2008; Choi et al., 2008; Zhang and Oyanedel-Craver, 2013, 2012).

The objective of this work is to compare batch and chemostat systems to assess the toxicity of casein-coated AgNPs on \textit{Escherichia coli} (\textit{E. coli}) based on membrane permeability, which is an indicator of membrane integrity and cells ability to adapt its
membrane lipid composition. The effects of AgNPs on *E. coli* have been widely studied, and thus the results produced in this research can be compared to those obtained previously. Spherical casein-coated AgNPs have been characterized and used in our research group and others (Zhang et al., 2012; Kvitek et al., 2009). To our knowledge, cell membrane changes due to nanoparticle exposure have not been studied and compared as a function of bacterial growth conditions, batch or continuous growth. The rate of substrate utilization and product formation are dependent on the growth conditions and can influence the nature and magnitude of the effect of AgNPs on the cell membrane, and thus, bacterial metabolism.

To gain additional mechanistic insight into AgNPs-membrane interactions, we have coupled bioreactor experiments with Langmuir film balance analysis of lipid monolayers formed using *E. coli* membrane extracts. Film balance studies have used to examine nanoparticle interactions with synthetic lipid monolayers, but they have not been used to examine lipid monolayers from membrane extracts to assess AgNPs exposure (Guzmán et al., 2013; Peetla and Labhasetwar, 2008; Torrano et al., 2013). Monolayer film balance analysis yields surface pressure-area isotherms that can be used to assess the biophysical properties of lipids as well as lipid composition (Kurniawan et al., 2013; Bothun et al., 2016; Venkataramanan et al., 2014). The influence of the bacteria growth conditions coupled with membrane permeability assays and membrane extract analysis provides new methodologies and testing conditions that may be used to more accurately examine the response of microorganisms to nanoparticle exposure.

### 3.2 Materials and Methods
3.2.1 Materials

A non-pathogenic strain of *E. coli* K-12 (ATCC 23716) was selected for this study. *E. coli* is a Gram-negative bacterium that has been extensively used in nanotoxicological studies (Venieri et al., 2014; Choi et al., 2008; Pratap Reddy et al., 2007). Reagents used to prepare the growth media for the bacteria — sodium chloride (NaCl), yeast extract, and tryptone —; and phosphate buffer solution (PBS) — monobasic potassium phosphate, dibasic potassium phosphate and Ethylenediaminetetraacetic acid (EDTA) — were purchased from Sigma Aldrich. The chemical oxygen demand (COD) was measured using the TNT 822 kit from Hach Company. Dichloromethane, methanol, and chloroform used for membrane extraction and Langmuir Blodgett tests were obtained from Sigma Aldrich. SYTO 9 and propidium iodide used for cell membrane permeation were purchased from Invitrogen. Standard casein-coated AgNPs were obtained from Argenol Company, Spain.

3.2.2 Methods

3.2.2.1 Nanoparticle characterization

Hydrodynamic diameter and zeta potential were measured using dynamic light scattering (DLS) (Malvern Zetasizer Nano ZS, ZEN 3600). Inductively-coupled plasma spectroscopy (ICP-OES optima 3100, Perkin Elmer) was used to measure the concentrations of AgNPs and Ag⁺ ions. Digestion in 2% nitric acid was required for each sample before analysis. The ionic release from AgNPs at each condition was quantified as per Liu and Hurt (2010) using centrifugal ultrafilter devices (ultra-4,3K) purchased from Amicon. Three concentrations of AgNPs — 1 mg/L, 15 mg/L and 50
mg/L — were used to assess the changes in permeation and surface pressure in chemostat and batch reactors.

### 3.2.2.2 Growth media and Bacteria culturing

Lysogeny Broth Miller (LB) growth media consisted of 10 g/L NaCl, 5 g/L yeast extract, and 10 g/L tryptone (Reddy et al., 2007). Culture media was immediately autoclaved after preparation. For each experiment, a fresh bacteria culture was grown for 12 hours in the LB media at 37°C. After that, optical density at a wavelength of 670 nm (OD$_{670}$) was measured separating the bacteria from the culture media by centrifuging it at 2500 rpm (1174 g) for 15 min and additional pellet resuspension in PBS (10%) solution (Zhang and Oyanedel-Craver 2012). PBS (10%) consisted of 1.12 g/L K$_2$HPO$_4$, 0.48 g/L monobasic potassium phosphate KH$_2$PO$_4$ and 0.002 g/L EDTA. The biomass concentration was measured indirectly through chemical oxygen demand (COD) using Hach TNT 822 with a Hach DR 2800 spectrometer. OD$_{670}$ was also used to follow the bacteria concentration evolution in each reactor. An optical density reading at this wavelength indirectly reflects the number of bacteria.

### 3.2.2.3 Bioreactors

A multiplexed chemostat arrays and a Synergy TM MX microplate reader (BIOTEK, VT) were used to perform the continuous and batch tests, respectively. All experiments were run in duplicate, including controls to detect contamination (media with no bacteria), non exposed condition (media plus bacteria) and exposed condition (media plus bacteria plus AgNPs). The ratio between number of AgNPs (estimated
from DLS size distribution measurements) and bacteria was kept constant for both chemostat and batch reactors to compare the bacteria response. However, it will change either through the influent of fresh media in the chemostat or growth of bacteria in the case of batch test.

### 3.2.2.3.1 Chemostats

Figure 6 shows a schematic of the multiplex chemostat arrangement. The bioreactors and sample vessels were sterilized twice before use. The array consisted of six small vessels fed with LB medium and air. Two needles were attached to each vessel for air and culture media injection. Airflow of 0.7 L/min was passed through a trap to remove humidity followed by a 0.2 μm filter and pumped into the bioreactors. The airflow maintained a DO of 0.6 mg/L inside the reactors. A peristaltic pump was used to feed the reactors with the culture media at a rate of 0.2 mL/min, setting up the dilution factor at 0.6 h⁻¹. Each bioreactor was inoculated with 300 μL of fresh bacteria solution. After 12.5 hours steady state conditions were achieved in terms of biomass concentration. Temperature and pH conditions at steady state were 37°C and a pH of 7.3 ± 0.3, respectively. After reaching stable conditions, AgNPs were injected to achieve the desired concentrations inside the vessel. Separate experiments were run with 1 mg/L, 15 mg/L and 50 mg/L of AgNPs. OD₆₇₀ and COD were used to follow the evolution of the bacteria concentration after the disturbance.

Samples were taken from each reactor every 2.5 hours using the sample needle (Figure 6). pH was measured immediately after sample collection. The OD₆₇₀ was measured using a spectrometer once the culture media had been removed from the
3.2.2.3.2 Batch tests

Batch tests were run using a microplate at similar conditions than those used during the chemostat tests. For both tests the ratio of bacteria/nanoparticle was kept constant. A 10 µL of bacteria stock solution was inoculated into each of the six wells containing 5 mL of LB media. After approximately 7.5 hours, when the culture reached log phase with an OD$_{670}$ was around 1 then AgNPs were injected to achieve the desire concentrations of 1 mg/L, 15 mg/L and 50 mg/L. After the injection of the nanoparticles, the plates were incubated for 5 hours to assess the AgNPs effect on

Figure 6: Schematic arrangement of chemostat reactor. Numbers in the figure correspond to each part of the array as follow: 1) sterile media carboy, 2) culture media lines, 3) multiplexed peristaltic pump, 4) port manifold, 5) airlines, 6) air dehumidifier, 7) pump, 8) collection bottles for samples, 9) collection bottles for controls, 10) vessel with ports.
bacteria concentration. OD$_{670}$ was determined using the same procedure described in
the chemostat section.

3.2.2.4 Langmuir film balance

The membranes of the bacteria were extracted before injection of the AgNPs
and after 5 hours of exposure to the respective nanoparticle concentration in the
chemostat. In this case, the batch tests samples collected at 5 hours were used for the
extraction of the membrane. After bacteria membranes were extracted according to
the protocol of Bligh and Dyer (Bligh and Dyer, 1959) lipid monolayers were
analyzed by Langmuir film balance based on the surface pressure-area isotherms.

Lipid packing, based on the total area occupied by the lipid extract at the
air/water interface at constant temperature, was altered with moveable barriers in a
Langmuir film balance. All results are presented for compression isotherms. Surface
pressure was calculated as $\pi = \gamma_o - \gamma$ where $\gamma_o$ is the air/water interfacial tension and $\gamma$
is the air/water/membrane extract (lipid) interfacial tension. The surface tensions were
measured using a Wilhelmy plate (Figure 7).

![Figure 7: Langmuir-Blodgett system including the main parts: barrier, trough, monolayer, and Wilhelmy plate.](image-url)
3.2.2.5 Epifluorescence staining membrane integrity test

A membrane integrity test was also performed for the batch reactors to compare the permeability membrane and the surface pressure changes. The cell membrane permeation of *E. coli* was determined using the Backlight kit (propidium iodide and SYTO 9) with a microplate reader. Propidium iodide becomes intercalated to the DNA within cells, and indicates whether bacteria that have a damaged membrane while SYTO 9 indicates intact cell membranes (Boulos et al., 1999). A stain solution composed of SYTO 9 and propidium iodide fluorescent nucleic acid stains was mixed at a 1:1 (v/v) ratio with a subsequent dilution in DI water (12μL of stain mixed solution in 2 mL of DI water). 100 μL of bacteria samples from each of the six wells with a fixed OD$_{670}$ of 0.06 were added to the wells of a 96 well flat-bottom black microplate. Thereafter, 100 μL of mixed stain solution was added and mixed thoroughly by pipetting at least 10 times for each well. Before reading with the microplate, 15 min of additional incubation was required in the dark at room temperature. A calibration curve of live and dead bacteria was necessary to quantify and compare the membrane disruption on bacteria before and after nanoparticle exposure. Each plate contained triplicate wells for each condition, and each plate was run in duplicate to quantify the Undisturbed Cell Membrane (UCM). The UCM (Eq. 1) is the green/red fluorescence ratio between bacteria exposed to AgNPs and the blank bacteria (bacteria not exposed to AgNPs) at given AgNPs concentration. Data was analyzed after 5 hours of AgNPs exposure to quantify inhibitory effect of AgNPs on the bacteria.
\[
\text{UCM} = \frac{P_t}{P_c} \times 100
\]

……Eq. 1

Where,

\(P_t\) = Green/red fluorescence ratio for bacteria exposed to AgNPs

\(P_c\) = Green/red fluorescence ratio for bacteria control without AgNPs

### 3.3 Results

#### 3.3.1 Physicochemical characterization

Characterizations of AgNPs consisted of size measurements in fresh media and bacteria-free media collected after 12 hours of bacterial growth (used growth media). The used growth media reduced the AgNPs stability producing aggregation. A concentration of 15 mg/L AgNPs was the more suitable concentration to measure size, using the DLS, because 1 mg/L was too close to the lower detection limit and 50 mg/L was above the ideal range of the instrument. Figure 8 shows the average hydrodynamic diameter of the AgNPs suspended in different media between 0 hours and 9 hours for 15 mg/L of AgNPs. It is expected that at 50 mg/L higher sizes of aggregate will be formed in comparison to 15 mg/L due the greater number of nanoparticles in the solution, thus increasing the nanoparticle-nanoparticle interaction and, therefore, the possible aggregation of the nanoparticles. On the other hand, at 1 mg/L nanoparticles average size is expected to be similar to the average size obtained at 15 mg/L.

Controls of AgNPs suspended in DI water showed that nanoparticles were stable at a size of 44.8±1 nm and zeta potential measurements ranging between -29.7 mV and -31.7 mV. Once AgNPs were contacted with LB media, the size increased to 73.51±19 nm. Likewise when AgNPs were contacted with used growth media
aggregation of nanoparticles was detected. The size of AgNPs increased from 86 nm at time zero to 282 nm after 5 hours showing that exopolymeric substances (EPS) released during the bacterial growth (composed of lipids, proteins and nucleic acids) can affect the AgNPs stability and could influence the inhibitory effect. Joshi et al., 2012 showed that EPS can trap the AgNPs outside the membrane as protective mechanism and decrease the inhibitory effect on bacteria due to the less interaction between bacteria and nanoparticles.

The dissolution of ions over time at room temperature was 1.2±0.2 % of the total silver concentration for 15 mg/L in DI water.

Figure 8: AgNPs stability in DI water, culture media and supernatant after 9 hours for 15 mg/L of AgNPs. Time measurements were taken at 0 hours, 2.5 hours, 5 hours and 9 hours for batch experiments. White markers represent AgNPs suspended in DI water, gray markers AgNPs suspended in fresh culture media and black markers correspond to AgNPs suspended in bacteria-free media collected after 12 hours of E. coli growth

3.3.2 Growth conditions

3.3.2.1 Chemostat

Bacteria grew for 12.5 hours (750 min) until steady state conditions were reached in terms of OD_{670}. At that time, AgNPs were injected only into the test bioreactors to achieve the desired concentrations (1 mg/L, 15 mg/L or 50 mg/L corresponding approximately to 7:1, 105:1 and 350:1 AgNPs/bacteria ratios,
respectively). Two bioreactors were used as controls, which contained bacteria without nanoparticles as controls to compare the growth rates between bacteria with and without exposure to nanoparticles, and two with only growth medium to detect possible contamination, and to study the interaction of AgNPs in the LB medium. Figure 9 shows the time when AgNPs were injected with a red arrow. Afterwards, the systems were operated for at least 5 additional hours (300 min). The OD$_{670}$ was measured to estimate bacteria concentration every 2.5 hours (150 min).

Only when the concentration of nanoparticles inside the chemostat was 50 mg/L a reduction on bacteria concentration was detected. In this case, a sustained decrease in OD$_{670}$ (16.3%) and COD (27.6%) after AgNPs injection time was detected after 12.5 hours. OD$_{670}$ increased after AgNPs injection time due to the nanosuspension brownish coloration at high AgNPs concentration. In the case of 15 mg/L a reduction on 11% of OD$_{670}$ and 8% of COD were measured after 5 hours (300 min) of the AgNPs injection. However, recovery of the bacteria concentration was detected to similar OD$_{670}$ and COD concentrations than those obtained before the injection of nanoparticles. No changes in terms of OD$_{670}$ or COD were found at 1 mg/L AgNPs. These results showed an inversely correlation between AgNPs and bacteria concentrations.
Figure 9: Growth of *E. coli* exposure to three AgNPs concentrations: a) 1 mg/L, b) 15 mg/L and c) 50 mg/L in the chemostat reactors. △ represents bacteria control without AgNPs. ▲ represents bacteria control exposed to AgNPs. ● represents a LB media control to detect contamination. □ represents LB media exposed to AgNPs to study nanoparticles stability and control to quantify the OD$_{670}$ from the AgNPs. The arrow and the red line show the time when AgNPs were injected into the system. OD$_{670}$ was read every each 2.5 hours (150 min) after samples were re-suspended in PBS 10%. Bars represent the error between duplicates.

AgNPs concentration was quantified after the injection in each reactor using the ICP-OES. Figure 10 shows that the AgNPs were inside the bioreactors between 5
hours (300 min) and 7.5 hours (450 min) after AgNPs injection for all conditions, when 0 hours corresponds to the time right before the AgNPs injection.

Figure 10: Average changes of AgNPs concentration inside of the chemostat bioreactors 12.5 hours after AgNPs injection a) 1mg/L, b) 15mg/L and c) 50mg/L. Zero time (0 hours) corresponds right before AgNPs injection. Data based on duplicate experiments.

3.3.2.2 Batch
Bacteria grew for 7.5 hours (450 min) until log phase was achieved. After that, AgNPs were injected to reach the required concentrations (1mg/L, 15mg/L and 50 mg/L) inside the microplate wells. Then, the test run for additional 5 hours (300 min), which corresponds to hydraulic retention time (HRT) in the chemostat reactor (Figure 11).

Nevertheless, AgNPs concentration of 50 mg/L was not suitable for the batch tests due to the fast aggregation and sedimentation leading to false readings in the results. Details are presented in detail in the SI.

The AgNPs inhibition on bacteria measured through the ratio of slopes based on OD<sub>670</sub> growth curves between bacteria exposed to AgNPs and the control bacteria (bacteria not exposed to AgNPs) were 20% and 0%, at 1 mg/L, and 15 mg/L of AgNPs, respectively. These values showed that 1 mg/L was the condition that produced a slight reduction in terms of bacteria concentration, compared to the other conditions.

The EPS most likely accumulated inside the batch reactors (microplate wells) promoting destabilization and sedimentation of the nanoparticles on the bottom of the well. Furthermore, less interaction between AgNPs and bacteria at 15mg/L due to high level of aggregation and sedimentation of AgNPs, can be the reason for the null or slight inhibitory effect detected. Additionally, AgNPs and the silver ions can be either chelated or coated by the EPS preventing the Ag<sup>+</sup> release. Figure 12 showed that EPS decrease the release of ions between 8 and 12 times for 50 and 15 mg/L (1 mg/L was not suitable for analysis because the concentration of ions was close to the ICP-OES detection limit) respectively, in comparison to Ag ions released into the culture media.
Figure 11: Growth of *E. coli* in exposure to two AgNPs concentration: a) 1 mg/L, and b) 15 mg/L in the batch reactors. White triangles show the growth curve of bacteria growing in a AgNPs free culture, while black triangles show the growth curve of bacteria exposed to the respective concentration of AgNPs. The red arrow and line show the time of injection of the AgNPs into the system. OD<sub>670</sub> was read each 15 min.
Figure 12: Percentage of silver ions released from AgNPs at concentrations of 50 mg/L and 15 mg/L suspended in freshly prepared culture media (black bars) and bacteria-free media collected after 12 hours of *E. coli* growth (white bars). Values were collected after 5 hours of AgNPs-media contact time at 37°C. Samples run in duplicate.

**3.3.3 Surface pressure analysis of membrane extracts**

**3.3.3.1 Chemostat**

Surface pressure was calculated as $\pi = \gamma_0 - \gamma$ where $\gamma_0$ was the air/water interfacial tension and $\gamma$ is the air/water/membrane extract (lipid) interfacial tension. Increases in $\pi$ in the presence of membrane lipids reflect a reduction in interfacial tension due to the surface-active properties of the lipids. Surface pressure-area ($\pi$–$A$) isotherms were measured with compression, or decreasing $A$, at a constant mass of membrane lipids. By holding the lipid mass constant, differences in $\pi$ between cell culture experiments with and without AgNPs can be attributed to changes in lipid composition.

Figure 13 shows results for $\pi$–$A$ isotherms conducted on membrane lipids obtained from chemostat cultures at AgNPs concentrations of 1 mg/L, 15 mg/L, and 50 mg/L. The increases in $\pi$ upon compression reflect a decrease in interfacial tension. At 1 mg/L and 15 mg/L the $\pi$–$A$ isotherms where cells were exposed to AgNPs were
shifted to lower areas relative to the control. In these cases, the lipid monolayers required more compression to achieve comparable surface pressures to the control.

This result infers that the membrane lipids where cells were exposed to AgNPs were occupied less area at the air/water interface. The most plausible explanation for this observation is that the lipids from extracts where cells were exposed to AgNPs contained more saturated than unsaturated acyl tails. Lipids with saturated acyl tails do not exhibit “tail kinking” and occupy less interfacial area than lipids with unsaturated acyl tails do. At 50 mg/L there was no statistical difference in the $\pi$–A isotherms with and without AgNPs exposure. These results suggest that in chemostat cultures membrane disruption caused by AgNPs was a destabilizing or fluidizing (disordering) effect where the cells responded by shifting their lipid composition to more rigid, saturated lipids to counteract membrane fluidization.

3.3.3.2 Batch reactors

3.3.3.2.1 Langmuir Blodgett

Figure 13b shows the results for $\pi$–A isotherms conducted on membrane lipids obtained from batch cultures. At 15 mg/L and 50 mg/L, the $\pi$–A isotherms where cells were exposed to AgNPs were shifted to higher areas relative to the control. In contrast to the results for membrane lipids from chemostat cultures, the lipid monolayers required less compression to achieve comparable surface pressures to the control, while the membrane lipids from the batch cultures occupied more area at the air/water interface. At 1 mg/L there was no difference in the $\pi$–A isotherms with and without AgNPs exposure. These results suggest that in batch cultures membrane disruption caused by AgNPs was a stabilizing or rigidifying (ordering) effect where the cells
responded by shifting their lipid composition to more unsaturated lipids to counteract membrane rigidification.

Direct comparisons for membrane lipid monolayer behavior between chemostat and batch cultures are made based on the change in area, \( \Delta A = A_{\text{AgNPs}} - A_{\text{control}} \), at 5 mN/m (Figure 13c). For both conditions, \( \Delta A \) increases within increasing AgNPs concentration. The key difference between these conditions is that at 1 mg/L AgNPs there is little change for the chemostat cultures, while for batch cultures the greatest change was observed.

![Graphs](image)

Figure 13: Surface pressure-area isotherms (\( \pi-A \)) of membrane lipid extracts from (a) chemostat and (b) batch cultures exposed to nanoparticles at 1 mg/L, 15 mg/L, and 50 mg/L. Error bars represent average standard error for duplicate experiments. (c) Change in area as a function of nanoparticle concentration at 5 mN/m relative to the controls.

### 3.3.3.2.2 Epifluorescence staining membrane integrity test

Undisturbed cell membrane (UCM) results are displayed in Figure 14. Live/Dead experiments detected a very low inhibitory effect of AgNPs on *E. coli* after 5 hours. The results indicated no significant membrane permeation of the cell for AgNPs concentrations of 50 mg/L and 15 mg/L with UCM of 100 and 97%, respectively. The previous results indicated minimal to no physical damage to *E. coli* between 15 mg/L and 50 mg/L of AgNPs. Only at low concentrations of AgNPs (1
mg/L) was a slight inhibitory effect (UCM= 90.7%) observed in bacteria. AgNPs aggregation at high concentrations and steric forces decrease due the EPS released during bacteria metabolism can decrease the AgNPs inhibitory effect on bacteria. Using t test, it was found a statistical difference between 1mg/L and 50 mg/L (p=0.026) and between 1mg/L and 15 mg/L, (p=0.027), however no statistical difference was found between 15 mg/l and 50 mg/L (p=0.152). The statistical analysis confirmed that low concentrations are a higher inhibitory effect in comparison with the high concentrations.

![Graph showing UCM vs AgNPs concentration](image)

Figure 14: Represents the undisturbed cell membrane of *E. coli* under AgNPs. Undisturbed cell membrane values were calculated for three concentrations of AgNPs, 1 mg/L, 15 mg/L and 50 mg/L at 5 hours. Each value represents an average of 4 wells from two duplicate plates.

### 3.4 Discussion

This study compared the use of chemostat and batch reactors to assess the casein coated AgNPs exposure to *E. coli* in terms of bacteria growth and surface tension changes. Conditions from chemostat reactors were replicated in the batch reactor to compare AgNPs inhibition effects in both systems.
In our study, it was found that there is only a small or null inhibitory effect at all the AgNPs concentration used in batch conditions. Therefore, these results differ from previous studies simulating natural water conditions (Zhang and Oyanedel-Craver, 2013; Mirzajani et al., 2011). This may be due that bacteria are growing condition (rather than non-growing in tests using natural water conditions) and a lower nanoparticle-bacteria ratio used in comparison to other studies (in other studies the same concentration of nanoparticles were added at the start of the batch culture were numbers of bacteria are very low). In addition, the EPS accumulation inside the bioreactors greatly affected the stability of AgNPs thus reducing the cell-nanoparticle interactions. The slight or null effect of AgNPs exposure to bacteria in batch cultures was also consistent with the membrane lipid monolayer results where AgNPs had a stabilizing effect on the E. coli membranes. Therefore batch conditions are not appropriate to evaluate the effect of nanoparticle exposure to bacteria for growth conditions.

In the case of chemostat reactors, the constant feeding and aeration seemed to increase the stability of nanosuspension compared to batch conditions. Constant inflow of fresh media could reduce the accumulation of EPS inside the reactor, while aeration could reduce the aggregation of nanoparticles and therefore increasing the contact between AgNPs and bacteria.

Greater inhibitory effect of AgNPs observed in chemostat cultures was consistent with the membrane lipid monolayer results that showed that AgNPs had a destabilizing effect which forced the bacteria to counteract this effect by synthesizing lipids that are known to strengthen cellular membranes. Langmuir Blodgett and
epifluorescence staining membrane integrity test for batch conditions showed a similar
trend. Here, 1mg/L of AgNPs had a higher inhibitory effect on bacteria compared to
15 mg/L and 50 mg/L of AgNPs.

3.5 Conclusions

This study shows that chemostat systems can be used to provide a better and
more comprehensive assessment of the nanoparticle inhibitory effects on
microorganisms compared to batch systems at growing conditions. Langmuir Blodgett
was used to evaluate the surface tension changes in bacterial membranes, this test
provided additional information to the staining membrane integrity test. The formation
of EPS during the growth of E. coli influenced the level of the AgNPs effect on the
cell membranes, and therefore the bacterial growth. With an understanding of the fate
of nanoparticles in aqueous media, a more careful selection of appropriate
toxicological methodologies and testing conditions can be made. This will allow for
more accurate studies that measure the responses of microorganisms to the exposure
of nanoparticles.

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Comparative study between chemostat and batch reactors to quantify membrane permeability changes on bacteria exposed to silver nanoparticles

Nelson M. Anaya, Fatemeh Faghihzadeh, Nasim Ganji, Geoff Bothun, Vinka Oyanedel-Craver*

Supplementary Information

*E coli* membrane composition

SI 1: *E coli* membrane composition
**Batch tests at 50 mg/L**

The batch test at 50 mg/L was not suitable due to the fast aggregation and sedimentation of AgNPs in contact with the EPS. It seems initially from the figure SI 2, that the bacteria in contact with 50 mg/L of AgNPs are growing continually even faster than the bacteria without AgNPs. However, there is a mix of factors that can lead to this misconception. First, the epifluorescence test indicates that the all of the bacteria are alive at this condition (Figure 9). Second, the increase in terms of OD$_{670}$ corresponds to the AgNPs sedimentation, which can be corroborated by the well image that appears in the figure SI 2 but it does not correspond to bacterial growth. Additionally, the control (composed by EPS and AgNPs with 50 mg/L) showed an atypical behavior because the OD$_{670}$ did not keep the direct correlation in time, instead of that dramatically decreased.

SI 2: Growth of *E. coli* in exposure to 50 mg/L of AgNPs in the batch reactors. White triangles show the growth curve of bacteria growing in a AgNPs free culture, while black triangles show the growth curve of bacteria exposed to the respective concentration of AgNPs. The red arrow and line show the time of injection of the AgNPs into the system. OD$_{670}$ was read each 15 min.
SI 3: Internal Controls between EPS-AgNPs and culture media-AgNPs

Table S1: AgNPs: bacteria ratio

<table>
<thead>
<tr>
<th>AgNPs [mg/L]</th>
<th>Number of AgNPs/mL</th>
<th>Ratio AgNPs/bac</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>6.74E+09</td>
<td>7</td>
</tr>
<tr>
<td>15</td>
<td>1.01E+11</td>
<td>105</td>
</tr>
<tr>
<td>50</td>
<td>3.37E+11</td>
<td>350</td>
</tr>
</tbody>
</table>

# Bacteria

1.70E+10 bac/mL
CHAPTER 4

Use of a Multiplex system to quantify the response of *Escherichia coli* to nanoparticles exposure

By

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Is prepared for submission

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Abstract

Continuous bioreactors were used to study the response of *Escherichia coli* (*E. coli*) exposed to casein-coated silver nanoparticles (AgNPs). The chemostat array consisted of six small vessels (Multiplex system) fed with LB medium. *E. coli* K-12 was grown in LB media at 37°C at two dilution factors (DF) of 0.1 h⁻¹ and 0.6 h⁻¹ which corresponds to two hydraulic retention times (HRT) of 5 hours and 2.5 hours, respectively. Fifteen hours after achieving steady state, bacteria were exposed to a pulse of nanoparticles to achieve concentration inside the vessels of 15 mg/L and 50 mg/L of AgNPs. Phenotypic measurements performed included membrane permeability, and biomass quantification for seven hydraulic retention times in each reactor. In addition, Fourier transformed infrared (FTIR) analysis was added to gain mechanistic insight about AgNPs - cell interactions.

A higher inhibitory effect in terms of COD reduction and membrane permeation were detected at high AgNPs concentration (50 mg/L) and the low DF (0.1 h⁻¹). A higher contact time in the DF of 0.1 h⁻¹ implied more time of interaction between AgNPs and bacteria in comparison with the DF of 0.6 h⁻¹. FTIR results showed that protein and fatty acid region were the functional groups most affected also at the high AgNPs concentration and contact time. The results did not agree with previous studies with regard to the specific growth rate due to the different contact times that were achieved in the chemostats at the different dilution factors applied.

**Keywords:** Silver nanoparticles, FTIR, chemostat reactors
4.1 Introduction

Silver nanoparticles (AgNPs) are one of the most commonly used nanomaterials in consumer products and medical applications due to their antimicrobial properties (Kasaraneni et al., 2014; Schifman et al., 2015; Prabhu and Poulou, 2012; Li et al., 2008). Nevertheless, AgNPs antimicrobial activity can be compromised, due to bacteria capability to modifying their phenotype in response to stress agents by genetic mutation or mobile genetic material acquisition from another bacterium (Fraser and Kærn, 2009; He and Chen, 2010; Leenheer and Cogan, 2008a; De Gelder et al., 2008; Palmer and Kishony, 2013).

AgNPs, and silver ions (Ag\(^+\)) are stress agents that can cause changes in the permeability of the cell membrane, protein structure, respiration cycle, and DNA replication (Losasso et al., 2014; Manke et al., 2013; Eckhardt et al., 2013; Prabhu and Poulou, 2012; Rai et al., 2012).

Bacterial adaptation to Ag\(^+\) at the molecular level has been previously reported as three main mechanisms: Ag\(^+\) accumulation, efflux Ag\(^+\) pump and halide ions effect. TEM and EDX analysis have revealed that a bacteria Pseudomonas stutzeri strain (AG259) can be resistant to Ag\(^+\) stress through the formation and accumulation of dense metal deposits (Slawson et al., 1992; Silver et al., 1999). A second mechanism consist on the pump of Ag\(^+\) from the cytoplasm to the bulk and the hydrogen ions pumping in reverse direction occur due to the activation of sil genes, which includes nine sil genes (silA, silB, silC, silE, silF, silR, silS, silP ORF 105 and silAB ORF96) for both efflux system, SilCBA and SilP. Two periplasmic protein (SilE and SilF) act as a molecular chaperon and transport Ag\(^+\) to SilCBA to continue injection (Silver,
Finally, the role of halide ions in the silver resistance mechanism is to decrease the availability of Ag$^+$ through binding with halide ions, in culture media (Gupta et al., 2001).

Continuous systems (chemostat) have been previously used to study the bacteria response to stress conditions in terms of cell growth and adaptation. (King et al., 2006; Leenheer and Cogan, 2008b; Lin et al., 2012; Ziv et al., 2013; Miller et al., 2013; Gresham and Hong, 2015). Chemostats allow bacteria to grow in a defined, ideally constant and controllable set of physico-chemical conditions. Moreover, continuous cultures have the advantage that time-independent concentration (steady state) can be achieved and the contact time between stressor and bacteria can be manipulated changing the rate of supply of the limiting substrate reflected (flow rate). Previous studies have reported that organisms growing at slow specific growth rates could adapt easier to stress conditions than those growing at a faster rate. (Van Hoek et al., 1998).

To our knowledge stress-response to AgNPs has not been systematically evaluated using continuous cultures as function of concentration and contact times. The objective of this work is to elucidate the response of *Escherichia coli* (*E. coli*) to the exposure casein-coated AgNPs at different contact times using multiplex chemostat reactors. At steady conditions the specific growth rate is equal to the dilution factor (DF), which is the ratio between the flow rate of medium injected and the culture volume, and where lower DF corresponds to greater contact times in comparison with higher DF.
To gain additional mechanistic insight about AgNPs - cell interactions, we have included Fourier transform infrared (FTIR) analysis. The changes of specific functional groups in biomolecules can be related to different toxicological mechanism affecting bacteria exposed to AgNPs. The influence of different bacterial growth conditions coupled with FTIR technique can be used as a new approach to examine the response of microorganisms to nanoparticles exposure.

4.2 Materials and Methods

4.2.1 Materials

During this study a non-pathogenic strain of *E. coli* K-12 (ATCC 23716) was selected. *E. coli* is a Gram-negative bacterium that has been extensively used in nanotoxicological studies (Venieri et al., 2014; Choi et al., 2008; Pratap Reddy et al., 2007). Reagents used to prepare the growth media and phosphate buffer solution (PBS) for the bacteria were purchased from Sigma Aldrich: sodium chloride, yeast extract, tryptone; monobasic potassium phosphate, dibasic potassium phosphate, ethylenediaminetetraacetic acid (EDTA), respectively. The chemical oxygen demand (COD) was used to measure biomass using the TNT 822 kit from Hach Company. SYTO 9 and propidium iodide used for cell membrane permeation were purchased from Invitrogen. Standard casein-coated AgNPs were obtained from Argenol Company, Spain.

4.2.2 Methods

4.2.2.1 Nanoparticle characterization
Hydrodynamic diameter and zeta potential were measured using dynamic light scattering (DLS) (Malvern Zetasizer Nano ZS, ZEN 3600). Inductively-coupled plasma spectroscopy (ICP-OES optima 3100, Perkin Elmer) was used to measure the concentrations of AgNPs and Ag\(^+\) ions. Before the analysis of each sample a digestion in 2% nitric acid was required. The ionic release at each condition from the AgNPs was quantified as per Liu and Hurt (2010) using centrifugal ultrafilter devices (ultra-4.3K) purchased from Amicon. The two concentrations of AgNPs inside the vessel were used: 15 mg/L and 50 mg/L. AgNPs exposure was measured at two contact times (hydraulic retention time) using multiplex chemostat reactors.

4.2.2.2 Growth media and Bacteria culturing

Lysogeny Broth Miller (LB) growth media consisted of 10 g/L NaCl, 5 g/L yeast extract, and 10 g/L tryptone (Reddy et al., 2007). After the culture media was prepared it was immediately autoclaved. For each experiment, a fresh bacteria culture was grown for 12 hours in the LB media at 37°C. Then, bacteria was separated from the culture media by centrifuging it at 2500 rpm (1174 g) for 15 min and additional pellet resuspension in PBS (10%) solution (Zhang and Oyanedel-Craver, 2012). The PBS (10%) solution consisted of 1.12 g/L K\(_2\)HPO\(_4\), 0.48 g/L monobasic potassium phosphate KH\(_2\)PO\(_4\) and 0.002 g/L EDTA. The biomass concentration was measured indirectly through chemical oxygen demand (COD) using Hach TNT 822 with a Hach DR 2800 spectrometer after separating bacteria from culture media by centrifugation and followed resuspension in PBS (10%). The COD reduction is measured after the AgNPs injection. Since COD cannot differentiate between alive and dead biomass, it is very likely that a delaying in the detection of COD changes will be expected. An
optical density at a wavelength of 670 nm (OD\textsubscript{670}) was measured to follow the bacteria concentration evolution in each reactor. An optical density reading at this wavelength indirectly reflects the number of bacteria.

4.2.2.3 Bioreactors

Multiplexed chemostat reactors were used to perform the continuous tests. All experiments were run in duplicate, including controls, to detect possible contamination (media with no bacteria), non exposed condition (media plus bacteria) and exposed condition (media plus bacteria plus AgNPs).

The bioreactors and sample vessels were sterilized twice before use in these experiments. The array consisted of six small vessels fed with LB medium and air. Two needles were attached to each vessel for air and culture media injection (Figure 15). A volumetric flow of air of 0.7 L/min was passed through a trap to remove humidity. This was then followed by a 0.2 µm filter before being pumped into the bioreactors, which corresponds to centrifuge tubes of 50 mL. The airflow maintained dissolved oxygen (DO) of 0.6 mg/L inside the reactors. Positive pressure generated during the air injection is used to collect the effluent samples from the vessels into the collection bottles (Figure 15). A multi channel peristaltic pump was used to feed each reactor separately with the culture media at a constant rate for each of the six lines of 0.2 mL/min, and 0.1 mL/min setting up the DF at 0.6 h\textsuperscript{-1}, and 0.1 h\textsuperscript{-1} respectively. The flow rates (and therefore DF) were selected in order to avoid wash out into the reactors. Each bioreactor was inoculated with 300 µL of fresh bacteria solution. After 12.5 hours steady state conditions were achieved in terms of biomass concentration. Temperature was 37°С and pH conditions at steady state were 7.3 ± 0.3, and 8.3 ± at
0.6 h\(^{-1}\) and 0.1 h\(^{-1}\) DF, respectively. After reaching steady conditions, AgNPs were injected to reach the desired concentrations inside the vessel. Separate experiments were run with each pulse injection of 15 mg/L and 50 mg/L of AgNPs. The evolution of the bacteria concentration after the addition of the AgNPs was followed by \(\text{OD}_{670}\) and COD.

Samples were taken from each reactor every 0.5 hydraulic retention time (HRT) (2.5 hours and 5 hours for 0.1 h\(^{-1}\) and 0.6 h\(^{-1}\) DF, respectively) After sample collection the pH was measured immediately. The culture media was then removed from the sample by centrifugation. The bacteria pellet was resuspended in 7.5 mL of PBS (10%), for \(\text{OD}_{670}\) and COD measurements.

![Diagram of chemostat reactor](image)

**Figure 15:** Schematic arrangement of chemostat reactor. Numbers in the figure correspond to each part of the array as follow: 1) sterile media carboy, 2) culture media lines, 3) multiplexed peristaltic pump, 4) port manifold, 5) airlines, 6) air dehumidifier, 7) pump, 8) collection bottles for samples, 9) collection bottles for controls, 10) vessel with ports.

### 4.2.2.4 FTIR tests
Liquid samples were prepared for ATR-FTIR (Nicolet iS50 FTIR, Thermo Scientific) analysis by fixing the OD$_{670}$ to 0.75 using an UV–vis spectrophotometer (Genesis, 10UV, Thermo Scientific). Following this, bacteria were centrifuged at 13,000 rpm (18,894 g) for 10 minutes, and the supernatant was removed. The pellets were suspended in 10 μL of PBS 10%, and the suspension of bacteria with AgNPs was directly transferred onto the crystal surface (Gurbanov et al., 2015).

Spectra were the result of 256 scans with a resolution of 4 cm$^{-1}$ in the 4000 cm$^{-1}$ – 350 cm$^{-1}$ spectral range. The data was analyzed by Omnic software (Thermo Scientific) and processed using Matlab (Mathworks Software).

A unique FTIR spectrum is detected for bacteria and each of their components and vibration modes. The full spectra range is divided in three specific regions; nucleic acid (900 cm$^{-1}$ to 600 cm$^{-1}$), carbohydrates plus proteins (1800 cm$^{-1}$ to 900 cm$^{-1}$) and the fatty acid region (3300 cm$^{-1}$ to 2800 cm$^{-1}$); and each region is divided in specific vibration modes. AgNPs effect on bacteria is detected through the peak shifting and peak intensity analysis of specific functional groups in biomolecules which can be related to different toxicological mechanism (Al-Holy et al., 2006; AlRabiah et al., 2013; Arakawa et al., 2001).

### 4.2.2.5 Epifluorescence staining membrane integrity test

The cell membrane permeation of the *E. coli* was determined using a BacLight kit (propidium iodide and SYTO 9) with a TM MX microplate reader (BIOTEK, VT). Propidium iodide becomes intercalated to the DNA within cells, and indicates whether bacteria have a compromised membrane while SYTO 9 indicates intact cell membranes (Boulos et al., 1999). SYTO 9 stains all cells green and propidium iodide
can interact with DNA in cells with compromised membrane. A stain solution composed of SYTO 9 and propidium iodide fluorescent nucleic acid stains was mixed at a 1:1 (v/v) ratio with a subsequent dilution in DI water (12 μL of stain mixed solution in 2 mL of DI water). 100 μL of bacteria samples from each of the six wells with a fixed OD$_{670}$ of 0.06 were added to the wells of a 96 well flat-bottom black microplate. Thereafter, 100 μL of mixed stain solution was added and mixed into each well by thoroughly pipetting at least 10 times. Additional incubation was required in a dark at room temperature for 15 min before reading with the microplate. A calibration curve of live and dead bacteria before and after nanoparticle exposure was necessary to quantify and compare the membrane disruption on bacteria. Each plate was run in duplicate and contained triplicate wells for each condition to quantify the Undisturbed Cell Membrane (UCM). The UCM (Eq. 1) is the green/red fluorescence ratio between bacteria exposed to AgNPs and the blank bacteria (bacteria not exposed to AgNPs) at given AgNPs concentration. After each sample was collected, the data was analyzed (Eq. 1) to quantify inhibitory effect of AgNPs on the bacteria.

\[
\text{UCM} = \frac{P_t}{P_c} \cdot 100
\]

Where,
\[P_t = \text{Green/red fluorescence ratio for bacteria exposed to AgNPs}\]
\[P_c = \text{Green/red fluorescence ratio for bacteria control without AgNPs}\]

### 4.2.3 Statistical analysis

The results from each data set were analyzed with SAS statistical software, version 9.1.2. A generalized linear mixer model (GLIMMIX) was selected due to the response...
was not necessarily normally distributed. A p value of less than 0.05 was considered to indicate significant difference among different DF, and AgNPs concentrations.

4.3 Results

4.3.1 Nanoparticle characterization

Characterizations of AgNPs consisted of size measurements in fresh media and bacteria free media collected after 15 hours of bacterial growth (used growth media). The used growth media reduced the AgNPs stability, producing aggregation. A concentration of 15 mg/L AgNPs was the more suitable concentration to measure size, using the DLS, because 50 mg/L was above the ideal range of the instrument (1mg/L to 20 mg/L). Figure 16 shows the average hydrodynamic diameter of the AgNPs suspended in different media between 0 hours and 28 hours for 15 mg/L of AgNPs using the DF of 0.1 h⁻¹. It is expected that at 50 mg/L greater sizes of aggregate will be formed due the greater number of nanoparticles in the solution, thus increasing the nanoparticle-nanoparticle interaction.

AgNPs suspended in DI water were stable at a size of 55.3±1 nm and zeta potential measurements ranging between -29.6 mV and -32.3 mV. Once AgNPs were contacted with LB media, their size increased to 84±5 nm. Likewise when AgNPs were contacted with used growth media, aggregation of nanoparticles was detected. The size of AgNPs increased from 52 nm at time zero to 217 nm after 5 hours showing that exopolymeric substances (EPS) released during the bacterial growth (composed of lipids, proteins and nucleic acids) could affect the AgNPs stability and could influence the inhibitory effect. Joshi et al.,( 2012) showed that EPS can trap the AgNPs outside
the membrane as protective mechanism and decrease the inhibitory effect on bacteria due to the less interaction between bacteria and nanoparticles.

The dissolution of ions over time in the used media at room temperature was 0.068% and 0.055% of the total silver concentration from reactors with 50 mg/L and DF of 0.1 h\(^{-1}\) and 0.6 h\(^{-1}\), respectively. The ion release reduction showed that AgNPs and the silver ions can be either chelated or coated by the EPS, preventing the Ag\(^+\) release (Anaya et al., 2016).

Figure 16: AgNPs stability in DI water, culture media and supernatant after 28 hours for 15 mg/L of AgNPs. Time measurements were taken at 5, 10, 15 and 28 hours. White bars represent AgNPs suspended in DI water, gray bars AgNPs suspended in fresh culture media and black bars correspond to AgNPs suspended in bacteria-free media collected after 15 hours of \textit{E. coli} growth

4.3.2 Biomass concentration changes
AgNPs were injected only into the test bioreactors after steady conditions were reached to achieve the desired concentrations (15 mg/L or 50 mg/L). Afterwards, the systems were operated for 35 hours and 10 hours for the DF of 0.1 h\(^{-1}\) and 0.6 h\(^{-1}\), respectively. The OD\(_{670}\) was measured to track bacteria concentration and COD to quantify biomass reduction after bacteria separation from culture media by centrifugation.

For a DF of 0.1 h\(^{-1}\) and 15 mg/L of AgNPs a reduction of 2% COD was detected at 25 hours after the AgNPs injection. After that, COD reduction was not observed.

In the case of 0.1 h\(^{-1}\) and 50 mg/L there was a reduction of COD from 5 hours to 25 hours of 13%, after 25 hours the COD reduction was not more than 5.6%.

For the DF of 0.6 h\(^{-1}\), a small reduction (1.8%) in biomass concentration was detected with 15 mg/L of AgNPs at 2.5 hours and 5 hours, while at 50 mg/L a decrease in COD (biomass) was observed at 5 hours and 10 hours up to 14.5% (Figure 17).
Figure 17: COD reduction after 35 hours for 15 mg/L and 50 mg/L of AgNPs using two DF a) 0.1 h\(^{-1}\) and b) 0.6 h\(^{-1}\). White bars represent samples from the reactor injected with 15 mg/L and black bars with 50 mg/L. Zero time (0 hours) corresponds right before AgNPs injection. Data based on duplicate experiments.

4.3.3 AgNPs concentration

AgNPs concentration was quantified after the injection in each reactor using ICP-OES. Figure 18 shows that AgNPs interact with bacteria between 25 hours and 35
hours for the DF of 0.1 h\(^{-1}\), and 7.5 hours and 10 hours for the DF of 0.6 h\(^{-1}\) after AgNPs injection. Residence time of nanoparticles without cells was not measured directly, but in steady conditions, the solid retention time (with cells) is equivalent to the hydraulic retention time (without cells).

Figure 18: Average changes of AgNPs concentration inside of the chemostat bioreactors 7 HRT after AgNPs injection: a) DF 0.1 h\(^{-1}\) and b) DF 0.6 h\(^{-1}\). White triangles represent samples from the reactor injected with 15 mg/L and black squares with 50 mg/L. Zero time (0 hours) corresponds right after AgNPs injection. Data based on duplicate experiments.
4.3.4 FTIR results

The finger print (nucleic acid region) bands of *E. coli* are found in the region between 900 cm\(^{-1}\) and 600 cm\(^{-1}\), (SI Figures SI1a, SI2a, SI3a, and SI4a). Bands between 1800 cm\(^{-1}\) and 900 cm\(^{-1}\) show the presence of C-O-C and C-O vibrations in the sugar rings in various components of polysaccharides (carbohydrates plus proteins) (Figures SI1b, SI2b, SI3b, and SI4b). Region between 3300 cm\(^{-1}\) and 2800 cm\(^{-1}\) shows the spectra for the fatty acid region (Figures SI1c, SI2c, SI3c, and SI4c). Spectra are shown including the untreated *E. coli* profile and *E. coli* exposed to AgNPs for all of the regions.

A unique FTIR spectrum is detected for the bacteria treated with AgNPs at each 2.5 hours and 5 hours for the DF of 0.6 h\(^{-1}\) and 0.1 h\(^{-1}\), respectively. Each specific bond corresponds to a specific biomolecules at specific wavelengths which have been completely defined in previous studies (Al-Holy et al., 2006; AlRabiah et al., 2013; Arakawa et al., 2001)

Tables 3 to 8 show the definition of the spectral bands of ATR-FTIR spectrum of a bacterium. Values represent the average of samples run in duplicate including error values. The changes of specific functional groups in biomolecules are expressed in terms of shifting and peak intensity, which can be related to different toxicological mechanism affecting bacteria exposed to AgNPs. Bacteria exposed to 50 mg/L showed greater changes in functional groups in comparison with 15 mg/L in both DF.

Results summarized in table 11 showed that the main changes were found in the fatty acid and protein region. For the condition in which 0.1 h\(^{-1}\) dilution factor and 50
mg/L of AgNPs was applied, the untreated bacteria had the most intense peaks throughout the spectrum compared to the bacteria exposed for 35 hours. The changes for the fatty acid region were shifting due to the deformation of asymmetric vibration of (C=O) and the asymmetric vibration of (P=O). On the other hand, in the protein region were shifted the peaks for amide III.

For the 0.6 h⁻¹ dilution factor for 50 mg/L of AgNPs, the same trend as 0.1 h⁻¹ dilution factor and 50 mg/L of AgNPs was found, where the untreated bacteria had the most intense peaks throughout the spectrum compared to the bacteria exposed for 10 hours. Changes were detected only in the fatty acid region, a shifting in the symmetric vibration of CH₃ was found.

Table 3: Comparison of fatty acids region of untreated and exposed E. coli with AgNPs using ATR-FTIR for a DF of 0.1 h⁻¹ and 50 mg/L.

<table>
<thead>
<tr>
<th>Vibration Mode</th>
<th>E. coli untreated Wavenumber (cm⁻¹)</th>
<th>E. coli+AgNPs (5 hours) Wavenumber (cm⁻¹)</th>
<th>E. coli+AgNPs (15 hours) Wavenumber (cm⁻¹)</th>
<th>E. coli+AgNPs (25 hours) Wavenumber (cm⁻¹)</th>
<th>E. coli+AgNPs (35 hours) Wavenumber (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>v₃(CH₃)</td>
<td>2958.8± 0.0</td>
<td>2958.8± 0.0</td>
<td>2958.8± 0.0</td>
<td>2958.8± 0.0</td>
<td>2958.8± 0.0</td>
</tr>
<tr>
<td>v₃(CH₂)</td>
<td>2923.6± 0.2</td>
<td>2923.6± 0.2</td>
<td>2924± 0.3</td>
<td>2924± 0.3</td>
<td>2923.6± 0.2</td>
</tr>
<tr>
<td>v₃(CH₃)</td>
<td>2873.4± 0.0</td>
<td>2873.4± 0.0</td>
<td>2873.4± 0.0</td>
<td>2873.4± 0.0</td>
<td>2873.4± 0.0</td>
</tr>
<tr>
<td>v₃(CH₂)</td>
<td>2853.2± 0.4</td>
<td>2853.2± 0.4</td>
<td>2853.6± 0.0</td>
<td>2853.6± 0.0</td>
<td>2853.2± 0.4</td>
</tr>
<tr>
<td>&gt;CH₂ deform.</td>
<td>1453.6± 0.1</td>
<td>1454.1± 0.2</td>
<td>1454.1± 0.2</td>
<td>1453.6± 0.1</td>
<td>1454.1± 0.2</td>
</tr>
<tr>
<td>v₃(C=O)</td>
<td>1397.2± 0.1</td>
<td>1396.7± 0.2</td>
<td>1396.2± 0.2</td>
<td>1394.8± 0.0*</td>
<td>1397.2± 0.1*</td>
</tr>
<tr>
<td>v₃(P=O)</td>
<td>1230.4± 0.2</td>
<td>1232.3± 0.1</td>
<td>1232.8± 0.0</td>
<td>1230.4± 0.0</td>
<td>1233.3± 0.1*</td>
</tr>
</tbody>
</table>

*Peak was shifted to a different wavenumber, **Peak was not observed.

Table 4: Comparison of proteins region of untreated E. coli and exposed E. coli with AgNPs using ATR-FTIR for DF of 0.1 h⁻¹ and 50 mg/L.
Table 5: Comparison of fatty acids region of untreated and exposed *E. coli* with AgNPs using ATR-FTIR for a DF of 0.6 h^{-1} and 50 mg/L.

<table>
<thead>
<tr>
<th>Vibration Mode</th>
<th><em>E. coli</em> untreated Wavenumber (cm(^{-1}))</th>
<th><em>E. coli</em>+AgNPs (5 hours) Wavenumber (cm(^{-1}))</th>
<th><em>E. coli</em>+AgNPs (15 hours) Wavenumber (cm(^{-1}))</th>
<th><em>E. coli</em>+AgNPs (25 hours) Wavenumber (cm(^{-1}))</th>
<th><em>E. coli</em>+AgNPs (35 hours) Wavenumber (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amid A</td>
<td>3278.4± 0.0</td>
<td>3281.8± 0.1</td>
<td>3281.3± 0.2</td>
<td>3279.8± 0.0</td>
<td>3282.3± 0.1</td>
</tr>
<tr>
<td>Amid B</td>
<td>3098.6± 0.0</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Amid I</td>
<td>1628.1± 0.1</td>
<td>1633.9± 0.2*</td>
<td>1633.9± 0.0*</td>
<td>1629.6± 0.1*</td>
<td>1634.4± 0.0*</td>
</tr>
<tr>
<td>Amid II</td>
<td>1536± 0.0</td>
<td>1540.8± 0.1*</td>
<td>1538.9± 0.01</td>
<td>1535.5± 0.2*</td>
<td>1541.3± 0.2*</td>
</tr>
<tr>
<td>Amide III</td>
<td>1303.6± 0.1</td>
<td>1301.2± 0.0</td>
<td>1300.8± 0.0</td>
<td>1288.7± 0.1*</td>
<td>1300.8± 0.0*</td>
</tr>
</tbody>
</table>

*Peak was shifted to a different wavenumber, **Peak was not observed.

Table 6: Comparison of proteins region of untreated *E. coli* and exposed *E. coli* with AgNPs using ATR-FTIR for DF of 0.6 h\(^{-1}\) and 50 mg/L.
Table 7: Comparison of fatty acids region of untreated and exposed *E. coli* with AgNPs using ATR-FTIR for a DF of 0.1 h\(^{-1}\) and 15 mg/L.

<table>
<thead>
<tr>
<th>Vibration Mode</th>
<th><em>E. coli</em> untreated Wavenumber (cm(^{-1}))</th>
<th><em>E. coli</em>+AgNPs (2.5 hours) Wavenumber (cm(^{-1}))</th>
<th><em>E. coli</em>+AgNPs (5 hours) Wavenumber (cm(^{-1}))</th>
<th><em>E. coli</em>+AgNPs (7.5 hours) Wavenumber (cm(^{-1}))</th>
<th><em>E. coli</em>+AgNPs (10 hours) Wavenumber (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amid A</td>
<td>3281.8± 0.0</td>
<td>3285.6± 0.0*</td>
<td>3282.7± 0.0</td>
<td>3283.7± 0.0*</td>
<td>**</td>
</tr>
<tr>
<td>Amid B</td>
<td>3098.6± 0.0</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Amid I</td>
<td>1635.8± 0.0</td>
<td>1638.7± 0.0*</td>
<td>1635.3± 0.0</td>
<td>1634.9± 0.0</td>
<td>1634.9± 0.0</td>
</tr>
<tr>
<td>Amid II</td>
<td>1543.7± 0.0</td>
<td>1545.7± 0.0</td>
<td>1544.7± 0.0</td>
<td>1545.2± 0.0</td>
<td>1546.1± 0.0</td>
</tr>
<tr>
<td>Amide</td>
<td>1236.6± 0.0</td>
<td>1237.6± 0.0</td>
<td>1237.1± 0.0</td>
<td>1237.6± 0.0</td>
<td>1238.6± 0.0</td>
</tr>
</tbody>
</table>

*Peak was shifted to a different wavenumber, **Peak was not observed.*

Table 8: Comparison of proteins region of untreated *E. coli* and exposed *E. coli* with AgNPs using ATR-FTIR for DF of 0.1 h\(^{-1}\) and 15 mg/L.

<table>
<thead>
<tr>
<th>Vibration Mode</th>
<th><em>E. coli</em> untreated Wavenumber (cm(^{-1}))</th>
<th><em>E. coli</em>+AgNPs (5 hours) Wavenumber (cm(^{-1}))</th>
<th><em>E. coli</em>+AgNPs (15 hours) Wavenumber (cm(^{-1}))</th>
<th><em>E. coli</em>+AgNPs (25 hours) Wavenumber (cm(^{-1}))</th>
<th><em>E. coli</em>+AgNPs (35 hours) Wavenumber (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(v_a(CH_3))</td>
<td>2958.8± 0.1</td>
<td>2959.2± 0.3</td>
<td>2958.8± 0.2</td>
<td>2959.7± 0.1</td>
<td>2958.8± 0.2</td>
</tr>
<tr>
<td>(v_a(CH_2))</td>
<td>2923.6± 0.0</td>
<td>2924± 0.2</td>
<td>2923.6± 0.2</td>
<td>2924.5± 0.2</td>
<td>2923.1± 0.0</td>
</tr>
<tr>
<td>(v_s(CH_3))</td>
<td>2873.4± 0.2</td>
<td>2873.9± 0.3</td>
<td>2873.4± 0.2</td>
<td>2874.4± 0.2</td>
<td>2873.4± 0.2</td>
</tr>
<tr>
<td>(v_s(CH_2))</td>
<td>2853.2± 0.0</td>
<td>2853.2± 0.0</td>
<td>2853.2± 0.0</td>
<td>2853.2± 0.0</td>
<td>2853.7± 0.2</td>
</tr>
<tr>
<td>&gt;CH(_2) deform.</td>
<td>1453.6± 0.2</td>
<td>1454.1± 0.2</td>
<td>1454.1± 0.2</td>
<td>1454.5± 0.3</td>
<td>1454.1± 0.2</td>
</tr>
<tr>
<td>(v_s(C=O))</td>
<td>1397.2± 0.0</td>
<td>1397.2± 0.0</td>
<td>1396.7± 0.2</td>
<td>1397.2± 0.0</td>
<td>1397.2± 0.0</td>
</tr>
<tr>
<td>(v_a(P=O))</td>
<td>1230.4± 0.0</td>
<td>1230.8± 0.2</td>
<td>1232.3± 0.3</td>
<td>1229.9± 0.2</td>
<td>1231.3± 0.3</td>
</tr>
</tbody>
</table>

*Peak was shifted to a different wavenumber, **Peak was not observed.*
Table 9: Comparison of fatty acids region of untreated and exposed *E. coli* with AgNPs using ATR-FTIR for a DF of 0.6 h⁻¹ and 15 mg/L.

<table>
<thead>
<tr>
<th>Vibration Mode</th>
<th><em>E. coli</em> untreated Wavenumber (cm⁻¹)</th>
<th><em>E. coli</em>+AgNPs (5 hours) Wavenumber (cm⁻¹)</th>
<th><em>E. coli</em>+AgNPs (15 hours) Wavenumber (cm⁻¹)</th>
<th><em>E. coli</em>+AgNPs (25 hours) Wavenumber (cm⁻¹)</th>
<th><em>E. coli</em>+AgNPs (35 hours) Wavenumber (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amid A</td>
<td>3278.4±0.2</td>
<td>3279.4±0.1</td>
<td>3281.3±0.0</td>
<td>3281.3±0.2</td>
<td>3280.8±0.0</td>
</tr>
<tr>
<td>Amid B</td>
<td>3098.6</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Amid I</td>
<td>1628.1±0.0</td>
<td>1631.5±0.2*</td>
<td>1632.9±0.0*</td>
<td>1632.9±0.0*</td>
<td>1632.9±0.0*</td>
</tr>
<tr>
<td>Amid II</td>
<td>1536±0.0</td>
<td>1541.8±0.3*</td>
<td>1539.4±0.0</td>
<td>1542.3±0.2*</td>
<td>1540.4±0.0*</td>
</tr>
<tr>
<td>Amide III</td>
<td>1303.6±0.0</td>
<td>1295.9±0.1*</td>
<td>1300.8±0.2</td>
<td>1293±0.0*</td>
<td>1300.8±0.2</td>
</tr>
</tbody>
</table>

*Peak was shifted to a different wavenumber, **Peak was not observed.

Table 10: Comparison of proteins region of untreated *E. coli* and exposed *E. coli* with AgNPs using ATR-FTIR for DF of 0.6 h⁻¹ and 15 mg/L.

<table>
<thead>
<tr>
<th>Vibration Mode</th>
<th><em>E. coli</em> untreated Wavenumber (cm⁻¹)</th>
<th><em>E. coli</em>+AgNPs (2.5 hours) Wavenumber (cm⁻¹)</th>
<th><em>E. coli</em>+AgNPs (5 hours) Wavenumber (cm⁻¹)</th>
<th><em>E. coli</em>+AgNPs (7.5 hours) Wavenumber (cm⁻¹)</th>
<th><em>E. coli</em>+AgNPs (10 hours) Wavenumber (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vₐ(CH₃)</td>
<td>2960.2±0.2</td>
<td>**</td>
<td>2960.7±0.1</td>
<td>2963.1±0.1</td>
<td>2960.2±0.2</td>
</tr>
<tr>
<td>vₐ(CH₂)</td>
<td>2924±0.0</td>
<td>**</td>
<td>2924±0.0</td>
<td>2925±0.4</td>
<td>2924±0.0</td>
</tr>
<tr>
<td>vₐ(CH₃)</td>
<td>2874.9±0.0</td>
<td>**</td>
<td>2875.3±0.1</td>
<td>**</td>
<td>2874.4±0.0</td>
</tr>
<tr>
<td>vₐ(CH₂)</td>
<td>2852.7±0.2</td>
<td>2855.6±0.1</td>
<td>2853.2±0.0</td>
<td>**</td>
<td>2853.2±0.0</td>
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<tr>
<td>&gt;CH₂ deform.</td>
<td>1454.5±0.0</td>
<td>1455±0.3</td>
<td>1454.5±0.0</td>
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<td>vₐ(C=O)</td>
<td>1397.7±0.0</td>
<td>1399.1±0.2</td>
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<td>1399.1±0.2</td>
<td>1398.1±0.0</td>
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<tr>
<td>vₐ(P=O)</td>
<td>1236.6±0.0</td>
<td>1237.6±0.1</td>
<td>1235.7±0.0</td>
<td>1236.1±0.2</td>
<td>1235.7±0.0</td>
</tr>
</tbody>
</table>

*Peak was shifted to a different wavenumber, **Peak was not observed.
Table 11: Comparison of proteins region of untreated E. coli and exposed E. coli with AgNPs using ATR-FTIR for DF of 0.6 h\(^{-1}\) and 15 mg/L

<table>
<thead>
<tr>
<th></th>
<th>Vibration mode</th>
<th>Changes detected</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>DF 0.1 h(^{-1})</td>
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<tr>
<td>Fatty acid</td>
<td>Asymmetric vibration of (C=O)</td>
<td>Shifting</td>
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<tr>
<td></td>
<td>Asymmetric vibration of (P=O)</td>
<td>shifting</td>
</tr>
<tr>
<td></td>
<td>Symmetric vibration of CH(_3)</td>
<td>NF</td>
</tr>
<tr>
<td>Protein</td>
<td>Amide III</td>
<td>Shifting</td>
</tr>
</tbody>
</table>

NF* Changes were not detected

4.3.5 Epifluorescence staining membrane integrity test

Undisturbed cell membrane (UCM) results are displayed in Figure 19. Live/Dead experiments detected a very low inhibitory effect (97\% - 94\%) at 15 mg/L of AgNPs on E. coli for both DF. Higher membrane permeation was found when the cells were
exposed to AgNPs concentrations of 50 mg/L in comparison to 15 mg/L, the highest inhibition was found with a UCM of 80% at DF of 0.1 h\(^{-1}\). The UCM results indicated physical damage to \textit{E. coli} at higher exposure time to AgNPs in combination with a high concentration of AgNPs. Aggregation at high concentrations of AgNPs and steric forces decrease due the EPS released during bacteria metabolism can prevent the AgNPs inhibitory effect on bacteria (Anaya et al., 2016).
Figure 19: Represents the undisturbed cell membrane of *E. coli* under AgNPs. Undisturbed cell membrane values were calculated for 15 mg/L and 50 mg/L of AgNPs, a) DF 0.1 h$^{-1}$ and b) DF 0.6 h$^{-1}$. White bars represent samples from the reactor injected with 15 mg/L and black bars with 50 mg/L. Zero time (0 hours) corresponds right before AgNPs injection. Each value represents an average of 4 wells from two duplicate plates.

4.4 Discussion

Formation of EPS during the growth of *E. coli* influenced the level of the AgNPs effect on the cell membranes. EPS accumulation inside the bioreactors greatly affected the stability of AgNPs and inhibited the Ag$^+$ release, thus reducing the cell-nanoparticle interactions. However, biomass concentration and staining fluorescence tests confirmed that AgNPs produced inhibition in terms of COD mass reduction and disturbance in cell membrane permeation even though the EPS formation. A higher inhibitory effect were found at the high AgNPs concentration (50 mg/L) and the low DF (0.1 h$^{-1}$), these conditions implies that the contact time between bacteria and nanoparticles is higher in comparison to the high DF (0.6 h$^{-1}$). For the DF of 0.1h$^{-1}$, 13.6% of COD reduction and 22% of membrane permeation were detected at 50 mg/L of AgNPs in comparison to 1.9% reduction of COD and 7.3 % at 15 mg/L of AgNPs and the same DF. The same trend was observed for the DF of 0.6 h$^{-1}$, but with lower inhibitory effect, 1.9% of COD reduction and 7.7% of membrane permeation.

The FTIR results also showed that the spectral regions changed based on the dilution factor and the AgNPs concentration. FTIR results showed that AgNPs also could cause changes in the fatty acids, specifically -CH deformation, which can be correlated to the alteration in membrane permeability. FTIR results also suggested that membrane permeability changes can be due to the dehydration of phospholipids.
The most remarkable functional groups changes of the exposed *E. coli* with DF 0.1 h\(^{-1}\) and 0.6 h\(^{-1}\) were in the fatty acid and protein region. This could suggest that part of the common changes between these two DF were due to the damage of conformational/compositional alterations in some of the components of the protein structures that could be intracellular proteins or cell wall peptides (Jiang et al., 2010; Nadtochenko et al., 2005).

DF of 0.6 h\(^{-1}\) showed alterations in the fluidity of the lipids produced by the symmetric vibration of CH\(_3\), which have been analyzed previously (Hu et al., 2009; Nadtochenko et al., 2005). These impacts on methyl groups’ stretching may be caused by the ROS, which identify malfunctions in the respiratory chain enzymes and other membrane proteins and lipophospho-polysaccharids (Jiang et al., 2010).

FTIR results shows that AgNPs can impact the structure and function of proteins (Losasso et al., 2014; Rizzello and Pompa, 2014; Wigginton et al., 2010) and can damage nucleic acid molecules with a higher inhibition at high AgNPs concentration (Choi et al., 2008b; Graves et al., 2015). The effect of membrane alterations in the treated *E. coli* was high compared to the observed changes in proteins. These changes were enough to damage the outer cell membrane by AgNPs, which causes more entry of AgNPs into the cells could be one of the reasons that AgNPs have cytotoxic effect on *E. coli* (Arora et al., 2008; Wigginton et al., 2010; Zhang and Oyanedel-Craver, 2013b)

Statistical analysis confirmed that the effect of different AgNPs concentrations are significant in terms of the COD reduction (p<0.001) and permeation (p=0.0324) in comparison with DF, which was not significant with COD (p=0.8161) but significant
for permeation (p=0.0157). For all of the conditions, AgNPs concentration was the most influential variable and determined the magnitude of the exposure response. This is consistent with the results obtained previously, which showed higher inhibitory effect in conditions with high AgNPs concentrations. Also a strong correlation was observed when the combined effect between DF and AgNPs concentration was analyzed, which is very significant to assess permeation response (p=0.0013). A similar trend was observed in the high toxicity effect of AgNPs on *E. coli* when bacteria were in longer contact with high AgNPs concentration.

### 4.5 Conclusion

This study showed that chemostat systems can be used to evaluate the inhibitory effect of nanoparticles in continuous culture at different growth rate of bacteria. The results did not agree with previous studies with regard to the specific growth rate due to the different contact times that were achieved in the chemostats at different specific growth rates. With the current conditions, there is not control of the contact time and the specific growth rate and contact time effects are combined. Longer term exposure and chronic studies are suggested to separate the growth rate effect from the contact time.

AgNPs were more statistically significant in comparison with the DF, although nanoparticles instability produced by the interaction with the EPS substance.
FTIR provided additional information to differentiating toxic effects at intracellular levels, being the protein and the fatty acid groups the most affected at 50 mg/L of AgNPs.

Continuous bioreactors coupled with FTIR represent a toxicological technique that can be used to assess systematically the response of microorganisms at different bacterial growth conditions to stress conditions such as nanoparticles, providing a clear understanding about the inhibitory effect produced on each bacteria component.

ACKNOWLEDGMENTS

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Use of a Multiplex system to quantify the response of *Escherichia coli* to nanoparticles exposure

By

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Supplementary Information

Statistical analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>COD</th>
<th>Permeation</th>
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<tbody>
<tr>
<td>DF</td>
<td>0.8161</td>
<td>0.0157</td>
</tr>
<tr>
<td>AgNPs</td>
<td>&lt;0.001</td>
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</tr>
<tr>
<td>DF*AgNPs</td>
<td>0.8678</td>
<td>0.0013</td>
</tr>
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</table>

![Graphs](image-url)
Figure SI 1: ATR-FTIR spectra of a) 900-600 cm\(^{-1}\) b) 900-1800 cm\(^{-1}\) c) 2800-3300 cm\(^{-1}\) for a DF of 0.1 h\(^{-1}\) and 50 mg/L AgNPs

Figure SI 2: ATR-FTIR spectra of a) 900-600 cm\(^{-1}\) b) 900-1800 cm\(^{-1}\) c) 2800-3300 cm\(^{-1}\) for a DF of 0.6 h\(^{-1}\) and 50 mg/L AgNPs
Figure SI 3: ATR-FTIR spectra of a) 900-600 cm$^{-1}$ b) 900-1800 cm$^{-1}$ c) 2800-3300 cm$^{-1}$ for a DF of 0.1 h$^{-1}$ and 15 mg/L AgNPs
Figure SI 4: ATR-FTIR spectra of a) 900-600 cm\(^{-1}\) b) 900-1800 cm\(^{-1}\) c) 2800-3300 cm\(^{-1}\) for a DF of 0.6 h\(^{-1}\) and 15 mg/L AgNPs